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Chlorophyll ..... S. Aronoff 525

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Managed, edited and published at
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by
EDMUND H. FULLING

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THE BOTANICAL REVIEW

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CHLOROPHYLL

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INTRODUCTION

The purpose of this review is to summarize certain areas of existing information concerning the chlorophylls. No attempt has been made to include all publications relevant to these substances, and a number which are mentioned have been available only as abstracts. The latter will generally be acknowledged as such. Certain phases have been largely omitted, e.g., photosynthesis (especially kinetic studies of gas exchange, the path of carbon, and to some extent physico-chemical studies of fluorescence in living tissue), phototaxis and photoperiodism. These are too extensive as such, and only those parts of them are covered as are directly concerned with chlorophyll. Summaries of certain other phases of chlorophyll are available elsewhere, e.g., the chemistry of chlorophyll (a variety of reviews is available); analytical methods, their medicinal and industrial use (13*a*); and absorption spectra of chlorophyll and related compounds (13*a*).

[1] Contribution No. 132 from the Institute for Atomic Research and the Department of Botany, Iowa State College, Ames, Iowa. This document is based in part on work performed in the Ames Laboratory of the Atomic Energy Commission.

TYPES

There have been described, at present, the following chlorophylls: *a*, *b*, *c*, *d*, bacteriochlorophyll (*a*, *b* and *c*) and protochlorophyll (possibly *a* and *b*). Of these at least the first two may be isomerized to forms termed *a'* and *b'*. A material previously isolated from the pigments of higher green plants (241) and then termed "chlorophyll *c*" was later found (135, 242) to be an artifact. Of the three photosynthetic groups—green plants, purple sulfur bacteria, and green sulfur bacteria (157)—the photosynthetic pigment in the last group is not yet precisely known. A table by Cook (38) of the chlorophylls in different algae is given in Table I.

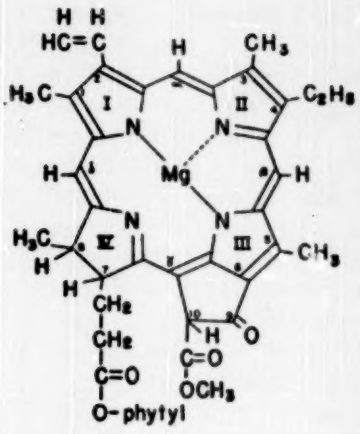
TABLE I

KNOWN DISTRIBUTION OF CHLOROPHYLLS IN ALGAE (FROM A. H. COOK)

	a	*b*	*c*	*d*
Chlorophyceae	+	+		
Xanthophyceae	+			
Chrysophayceae				
Bacillariophyceae (diatoms)	+	+	+	
Dinophyceae (dinoflagellates)	+		+	
Euglenineae	+	+		
Phaeophyceae	+		+	
Rhodophyceae	+			+
Myxophyceae	+			

The formulae and properties given for chlorophyll are of necessity those of isolated material. The extent to which they differ from the material as present in the cell is not adequately known; the nature of the difficulties will be indicated.

CHLOROPHYLLS *a* AND *b*. The material which is the predominant coloring of living matter on the surface of the earth is chlorophyll *a*. Its formula (I) differs from that of the generally accompanying chlorophyll *b* (II), as has long been known, by possessing a methyl instead of a formyl group in the 3-position. The normal approximate ratio of these pigments in higher plants is 3/1 (*a*/*b*). Their absorption spectra (Fig. 1) are now known with sufficient precision for all analytical work (134, 243).



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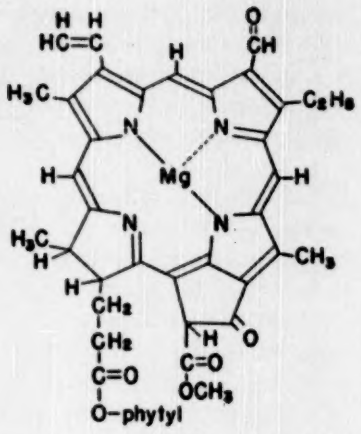


II

Seybold, Egle and Hulsbruch (196, 197) have extended their investigations for the chlorophyll contents of fresh-water algae and have come to the general conclusion that pigment investigations provide a sound basis for the examination of phylogenetic relationships in algae. Gessner (72) has indicated that the chlorophyll content of the plankton from Bavarian lakes provides a good measure of the production capacity of the corresponding water, a conclusion more or less previously reached by Manning and Juday (136) for Wisconsin lakes. Dutton and Juday (47) were unable to correlate chromatic adaptation of fresh-water plankton with depth distribution. The change in Palogloea from yellow in the upper zones to bright green in the 4–10 m. zone, which was the result of chlorophyll increase, carotenoid remaining constant, is explained as probably being due to a response to low light intensity rather than being that of a chromatic adaptation.

Inman (108) found that thermal algae (Myxophyceae) and *Chlorella* growing in temperatures of 37–72° C. had normal chlorophylls and photosynthesized not only at the growth temperatures but at colder values.

It is interesting to note that the parasitic mistletoe, *Phoradendron flavescens*, contains chlorophylls *a* and *b* and also photosynthesizes (68). The nature of the deficiency is, therefore, of interest. Presumably it is the inability of this plant to perform its root functions, i.e., obtain water and nutrients.

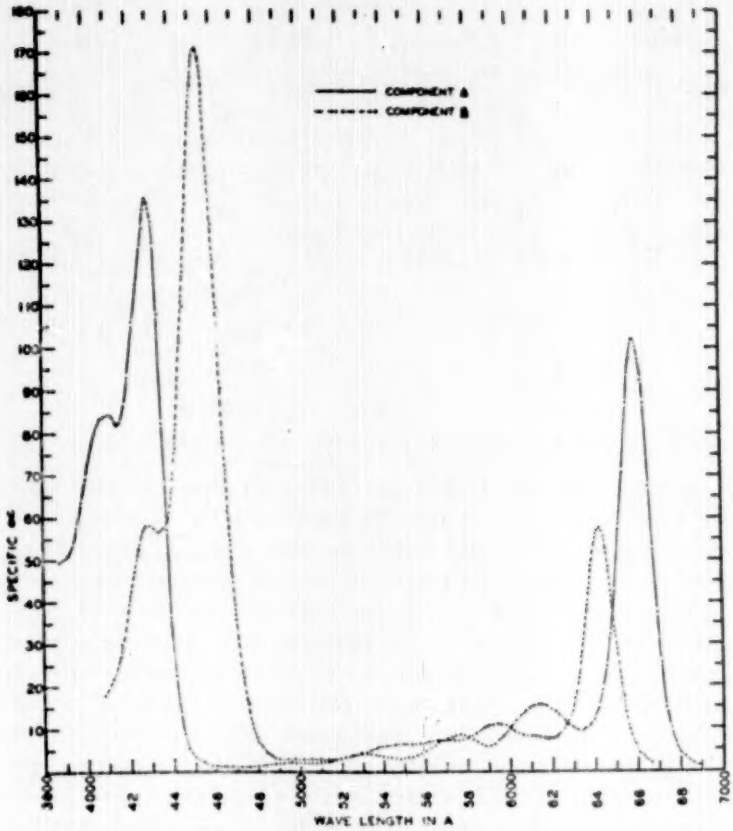


FIG. 1. The absorption spectra of chlorophylls *a* and *b* (from Zscheile and Comar (243)).

Of predominate interest in chlorophylls are the locations of hydrogens which may be active in photoreduction. The activity of the hydrogen on C–10 is well known. Among the more spectacular demonstrations of this is the keto-enol tautomerism, shown by the formation of a benzoyl derivative of the enol (219) and implied in the spectrum of phylloerythrin (13). To this carbon has also been ascribed the properties involved in the reversible leuco formation of chlorophyll (125) and various photochemical studies of chlorophyll discussed elsewhere. Furthermore, because of the ease

of isomerism of the pyrrolenin hydrogens (on 7,8) saturating the vinyl group and forming porphines, it is conceivable that these hydrogens may also be involved in photochemical reductions. A model reaction of this type is available in the photochemical oxidation of the Zn salt of the synthetic tetraphenyl chlorin of the corresponding porphine (15, 35).

Strain and Manning (220) have provided evidence of an isomerization of chlorophylls *a* and *b*, the isomers being termed, correspondingly, *a'* and *b'*. The isomers, in *n*-propanol, are interconvertible slowly at room temperature, rapidly at 100° (equilibrium mixture 4 *a* or *b* / 1 *a'* or *b'*). The isomers yield spectrally different pheophytins but similar pheopurpurins. They are made evident by chromatography upon dried confectioners' sugar, using petroleum ether–0.5% *n*-propanol–0.5% purified dimethylaniline as wash solvent. The *a'* appears below the *a*, the *b'* below the *b*. No explanation as to character of the isomerization is offered, except that, since different pheophytins are obtainable, the character of the Mg bonding is not involved. According to the present formula of chlorophyll, it is possible that isolation of the keto-enol tautomers have been accomplished, or even the cis-trans isomers originating from the positions of the hydrogens at 7,8. An additional possibility[2] is that of the separation of the chlorophylls into the geometrical isomers corresponding to the cis and trans forms in the phytol.

Seybold (189, 191), correlating the chemical compositions of sun- and shade-loving plants with that of the various algae which lack chlorophyll *b*, has come to the conclusion that the chlorophylls have different functions aside from that of photosynthesis. To chlorophyll *a* and its protein moiety he ascribes the formation of glucose and other monosaccharides, while chlorophyll *b* polymerizes the monosaccharides to form starch. This theory arises from the absence of *b* in those algae which do not form starch, and its presence in those which do. Why a similar variation does not occur in, e.g., young wheat plants, which do not form starch, and old ones, which do, is not considered.

CHLOROPHYLL *c* (chlorofucine, chlorophyll γ, chlorophyllin γ). This pigment, noted as long ago as 1873 by Sorby (215) and

[2] Manning, W. M. Personal communication.

believed to be an artifact by Willstatter and Page (233), though denied by Wilschke (232), has been thoroughly confirmed in investigations by Strain *et al.* (221). Using centrifuged pure cultures of diatoms and algae, extraction was accomplished with absolute methanol containing 0.2% dimethylaniline, which, after addition of water, was partitioned with petroleum ether, the xanthophylls and chlorophyll *c* remaining in the methanol layer. On further addition of water, additional contaminating pigment could be transferred to ether. With addition of fresh ether and excess salt solution to the aqueous layer the chlorofucine could be transferred to ether. A simpler method of preparation involves transfer of the total pigment complex to ether, concentration of the latter and its dilution with petroleum ether, followed by chromatography on powdered confectioners' sugar, the chromatogram being developed with petroleum ether–2–4% methanol. The chlorophyll *c* is described as forming a pale green band at the top of the column, underlain by fucoxanthin, while other materials, as chlorophyll *a*, and carotene pass through rapidly. The pigment prepared by the adsorption method possesses a spectrum somewhat different from that given for the partition method (Fig. 2), though the latter could be made to yield spectra more like the former by chromatography. It therefore appears that chlorophyll *c* either is still a mixture or is subject to changes, as oxidation or isomerization, on chromatography. More specifically, chlorophyll *c* is not equivalent to chlorophyll *a'*.

Granick (84, 85) has recently shown that chlorophyll *c* possesses Mg (removable by 4 *N* HCl), gives a phase test, lacks phytol and, following Mg removal, gives a porphyrin spectrum. He thus suggests that chlorophyll *c* is a Mg pheoporphyrin, more closely allied to protoporphyrin than to chlorophyll itself.

CHLOROPHYLL *d*. Using a procedure essentially similar to that given above, but taking advantage of greater ease of extraction, a new green pigment, chlorophyll *d*, has been found in red algae by Manning and Strain (137). Finding no chlorophyll *b* or *c* in these algae, separation was most readily effected after partial extraction by chromatography on powdered confectioners' sugar. The chlorophyll *d*, which is found above chlorophyll *a*, yields the spectrum given in Fig. 3. (The prominent band ca. 460 mμ would indicate a contaminant of some kind, especially since this is missing

in the corresponding pheophytins; this possibility is not indicated by the authors.) After ashing of a specimen, the metal in the center was shown colorimetrically by Titan yellow to be Mg.

As in the case of chlorophylls *a* and *b*, chlorophyll *d* is also found to isomerize in a bewildering number of manners. The pigment in methanol is converted into three other compounds, rapidly on heat-

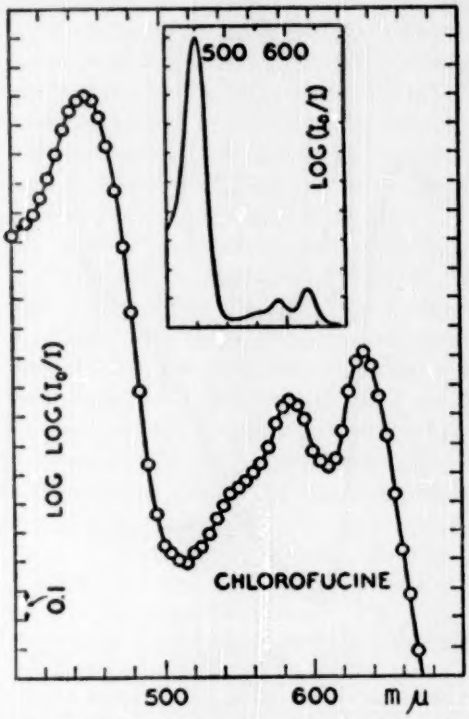


FIG. 2. The absorption spectrum of chlorophyll *c* (from Strain, Manning and Hardin (221)).

ing, slowly at room temperature. One of the three has a spectrum similar to that of chlorophyll *d* and is termed *d'*. The other two (all three being evident chromatographically) have different spectra, though resembling each other, and are termed isochlorophylls *d* and *d'*. The *d*'s and *d'*'s are interconvertible in the same manner as the *a*'s and *a'*'s. Furthermore, although by indirect evidence only, it is believed that isochlorophyll is interconvertible

with chlorophyll *d*. Addition of Mg grignard to a dry ethereal solution of isopheophytin *d* resulted primarily in isochlorophyll *d*, but it is stated that both *d'* and *d* were produced by isomerization of this synthetic isochlorophyll *d*. On the other hand, pheophytin *d* did not yield primarily chlorophyll *d* or the iso-compound, but a mixture of unknown materials. The type of pheophytin formed

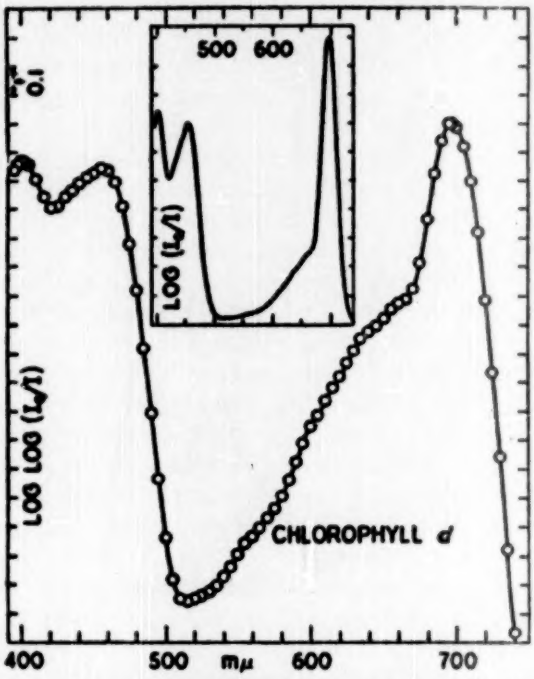


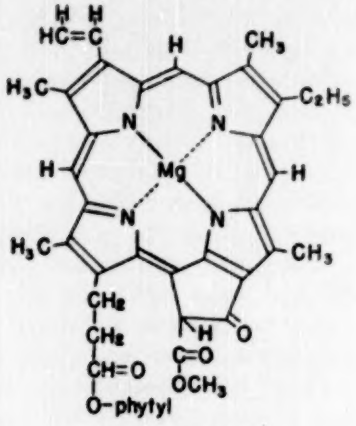
FIG. 3. The absorption spectrum of chlorophyll *d* (from Manning and Strain (137)).

appears to be a rate phenomenon, the compound produced at – 80° C. being pheophytin *d*; at room temperatures, isopheophytin *d*. Furthermore, at room temperature, by addition of acid, pheophytin *d* is rapidly converted to the iso-derivative. The iso-series thus appears to be the more "stable" series and to have spectral and adsorption properties similar to those of chlorophyll *a*. It is apparent that some organic chemistry remains to be done in the chlorophylls.

BACTERIOCHLOROPHYLLS. Bacteriochlorophyll is 3,4 dihydrochlorophyll *a*. Its preparation involves difficulties, primarily of oxidation, since extreme care must be used to avoid removal of two of the four hydrogens which form pyrrolenin rings. In a continuation of earlier work, Mittenzwei (144) has substantiated the previous tentative assignment of the additional two hydrogens in the 3,4 positions. The bacteriochlorophyll, isolated on the oxidized product 2 acetyl (2-desvinyl) chlorophyll *a*, could be reduced to the 2,α-oxy-meso derivative[3] and thence, by heating in a high vacuum, to the 2-vinyl, i.e., to chlorophyll *a* itself. The assignment of the two additional labile hydrogens was based on oxidations with chromic acid, from the basic fraction of which an optically active oil, α-methyl α′-ethyl succinic anhydride, was obtained. This could have come only from ring II or III and, because of the identity produced in the conversion of bacteriochlorophyll to chlorophyll *a*, is ascribed to ring II. Seybold and Egle (193) have treated *Thiocytis* bacteria in a manner similar to Strain *et al.* and have come to the conclusion that there is but one bacteriochlorophyll *a*, but that two others, *b* and *c*, are artifacts resulting from the method of preparation. The methanol extract of the bacteria was transferred to benzene to the extent to which pigment would enter. The solution was then concentrated under inert gas and the mixture chromatographed on powdered sucrose, resulting in a green bacteriochlorophyll *a* on top and a blue *a* below it. By salting the methanol solution, further pigment was forced into additional benzene, and a third pigment, bacteriochlorophyll *c*, obtained. Chlorophylls *b* and *c* had relatively similar spectra, with the main band in the red at 695–665 mμ, while that for *a* lay at 700–665 mμ, indicating a somewhat further maximum into the infra red. If the original bacterial solution was converted directly into pheophytin by extraction with methanol containing oxalic acid, only the pheophytin of *a* was obtained. If, however, a solution of pure *a* was allowed to stand in methanol for several hours and then converted to pheophytin, both *a* and *b* were obtained. It is thus presumed that bacteriochlorophylls *b* and *c* are artifacts. The close resemblance of these reactions to the isomerizations of Strain and Manning are obvious.

[3] Meso-compounds are those which contain two additional hydrogens, generally a saturated vinyl group. Meso-chlorins and phorbins thus possess the two hydrogens at 7 and 8, but contain a 2-ethyl rather than a 2-vinyl.

PROTOCHLOROPHYLL. The porphine nature of protochlorophyll (III) was first shown by Noack and Kiessling (161), and its definitive structure as the Mg chelate of the phytol-esterified vinyl



III

pheoporphine a_5 (61, 62) by Stoll and Wiedemann (219). The former workers also suggested that protochlorophyll is a precursor, rather than a degradation product, of chlorophyll. Seybold and Egle (192) are of the opinion that there is a protochlorophyll *b* as well as the corresponding protopheophytins. On the basis of spectral comparison with synthetic protochlorophyll this has been questioned by Fischer and Oestreicher (*loc. cit.*), as in the protochlorophyll (Fig. 4) isolated by Koski and Smith (118) from etiolated barley seedlings. The protopheophytins are probably artifacts of preparation, although they, too, were noted in the work by Koski and Smith. These authors also confirm the action spectrum of chlorophyll formation (67) which was the first positive proof of the origin of chlorophyll from protochlorophyll. At that time the action spectrum was somewhat at odds with the absorption spectral data of Seybold and Egle, but the latter are now known to be inaccurate.

THE NATURAL STATE

One of the most persistent problems in plant physiology is the nature of the existence of chlorophyll, whether it is bound to a

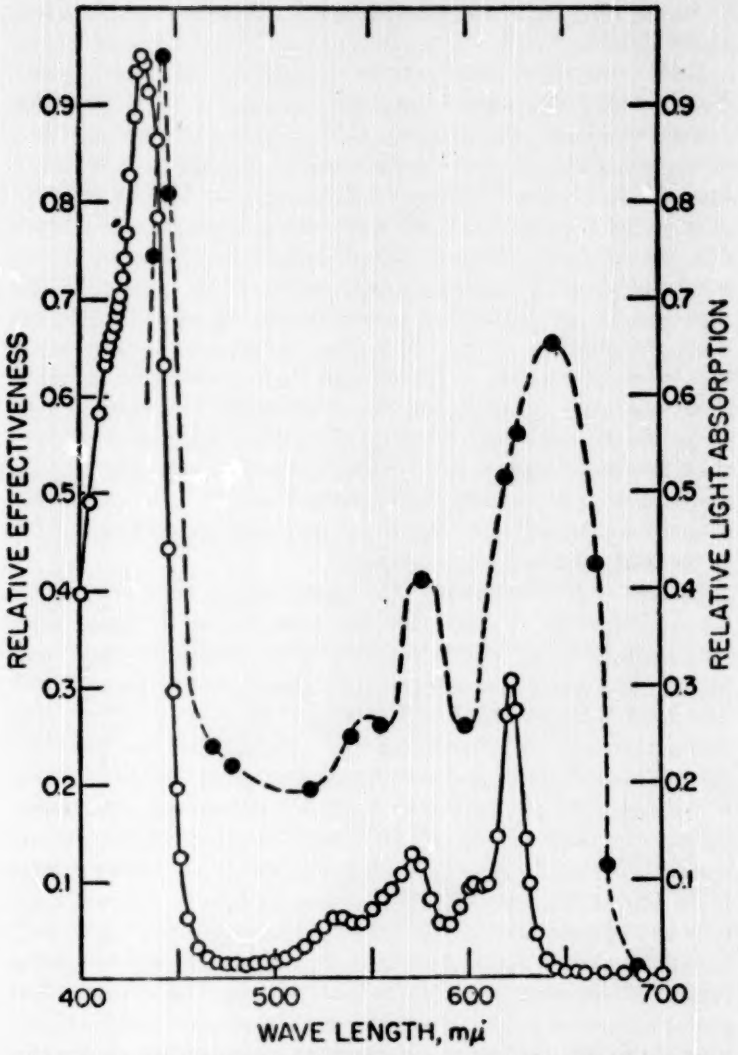


FIG. 4. A comparison of the action spectrum of chlorophyll formation (dotted lines) and the absorption spectrum of protochlorophyll (solid line) (from Smith (214)).

protein, whether it is present as a solid-liquid colloid, or dissolved in an oily matrix, or linked in a complex manner with a variety of substances.

It is of interest to summarize an analysis of a typical chloroplast. Menke (140) has shown that leaves average 17% chloroplasts. Using differential centrifugation (139, 83), he obtained essentially pure chloroplasts, analysis of the material showing it to be 47.7% lipid, 37.4% protein, 7.8% ash, 7.1% undetermined. Chibnall (36) gave as the analysis of the lipids of typical chloroplasts of cabbage (% of total ether extract): chlorophylls, 9.3%; carotene, 0.5%; xanthophyll, 0.8%; glyceride fatty acids, 17.5%; wax, 12.3%; sterols, 4.5%; undetermined unsaponifiable material, 13.3%; calcium phosphatide, 18.4%. It is thus apparent that the primary matter of chloroplasts is protein and that considerable matter is there in greater quantity than that of chlorophyll. Analyses similar to the above are also given by Bot (28). Hanson *et al.* (94) state that in young leaves chloroplast protein is only about 7% of the total protein but later increases to 35–40%. The chloroplast proteins are exceptionally rich in S, containing approximately 70% of the total protein S of the leaves.

Godnev and Kalishevich (77), among others, have investigated the concentration of chlorophyll per unit volume of tissue, using the chloroplasts of *Mnium medium* which possesses very small leaves. The average number of chloroplasts per leaf was 775,000. The total chlorophyll per leaf was (for 22 leaves) 1.91 γ. The average amount of chlorophyll per chloroplast was, therefore, $2.4 \cdot 10^{-6}\ \gamma$. We may extend the data of these authors further, using 4 μ as an average diameter for the chloroplasts, which then have a volume of $35\ \mu^3 = 35 \cdot 10^{-12}$ cm.^{3} In this volume then are the $2.4 \cdot 10^{-6}\ \gamma$ chlorophyll, which is equivalent to a concentration of $2.4 \cdot 10^6\ \gamma/35$ cm.$^3 = 72 \cdot 10^6\ \gamma$/liter $= 72$ g./l. This is, then, a molar concentration of $72/890 = 0.08$ or approximately 0.1 *M*. Calculations made with other plants by other authors [see the discussion of the above subject in Rabinowitch (180)] have given values as high as 0.2 *M*. Consider the spatial significance of such a concentration, assuming uniform distribution and neglecting, for the moment, the requirements of the phytol, concerning ourselves solely with the phorbin nucleus. On the basis of X-ray studies of the phthalocyanines, and our general knowledge of porphyrin

structure, we may safely assume an average diameter for the phorbin ring as somewhat greater than 10 Å. An isotropic distribution for the $12 \cdot 10^{22}$ molecules/liter in a 0.2 *M* solution results in a unit volume/molecule of 8 Å^3 (i.e., there are $1.2 \cdot 10^{23}$ molecules in 10^{24} Å^3). Obviously the concentration of 0.2 *M* is possible only if considerable packing occurs, so that the average distance between planes is less than that of their diameter. However, the phytol, which has heretofore been neglected, must assume a position between phorbin planes. It would thus appear that even a 0.1 *M* concentration of chlorophyll in the chloroplasts is an almost saturated solution. Inasmuch as the chlorophyll represents only a minor portion of the chemical species, the chloroplasts are thus

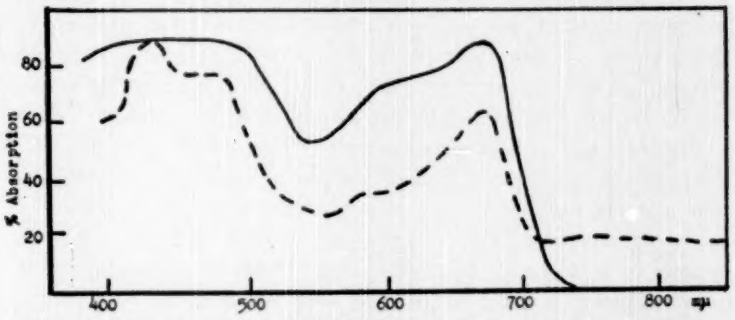


FIG. 5. Absorption spectrum of *Dolichos* leaf (solid line) and a suspension of chloroplasts from a *Dolichos* leaf (dotted lines) (adapted from Rabideau, French, and Holt (179)).

depicted as highly concentrated bodies, both the chemistry and physics of which are probably not deducible directly from observations on more dilute solutions. Under these conditions spectral observations, energy transmission, etc., assume different aspects than normal. Thus, although one of the common arguments in favor of the colloidal state of chlorophyll is the red-shifted spectrum of chlorophyll in leaves as compared to its solution in organic solvents, a condition which can be duplicated by synthetic colloidal preparations, it is well known that concentrated substances may often polymerize somewhat, resulting in spectral shifts. To what extent this accounts for leaf spectra is not known, and recent studies in this regard (Fig. 5) (107, 141, 179, 198) still do not take this point of view. Meyer, however, also believes that the

chlorophyll extracted from leaves by present methods yields a material which is not identical with that present as native chlorophyll, although his method, which does not differ radically from present procedures, does. Both Bot (28) and Seybold and Egle (194) have suggested that chlorophyll is present in leaves as two phases, one dissolved in lipids and the other bound to protein. Synthetic mixtures of this type have recently been made by Lepeschkin (124), which exhibit staining and solubility properties similar to those of chloroplasts. Following the suggestions of chlorophyll-protein combination by Lubimenko (131), Noack (159) succeeded in preparing material containing both precipitable protein and chlorophyll, and, more important, chlorophyll which fluoresced, a characteristic of undenatured (non-precipitated) material. Further, by various conditions which denatured the protein, the fluorescence of the chlorophyll disappeared, indicating that the chlorophyll was flocculated. There have been similar recent studies (3, 63, 64, 68, 149, 208–212) aside from those of Menke.

Fishman and Moyer have concerned themselves primarily with the electrophoretic-pH curves of various legumes and of *Aspidistra*. The material isolated from legumes, undoubtedly of a nature akin to the "grana" used by a number of workers, and probably containing by adsorption or combination a host of other substances besides chlorophyll and protein, showed an isoelectric region near the pH of 4.7 (0.02 *M* acetate buffer, 25°). The migration rate was independent of size, indicating that ionic charges, rather than zeta potential, were the predominating cause for electrophoretic migration. From the nature of the curves it was suggested that the proteins of the different legumes were all similar. In this regard some interesting experiments (116) were performed on colloidal chlorophyll in water which was shown to be negatively charged in a Zn-Cu couple. In a quartz cell with Pt electrodes, the particles, observed with an ultramicroscope, went to the anode, and reversing the current reversed the movement. With a potential difference of 1 v./cm. their speed was $30.3 \cdot 10^{-5}$ cm./sec.

The results, therefore, indicate particles of large state of aggregation. Bearing a negative charge, the particle could be flocculated by cations; the relative amounts of KCl, $MgCl_2$ and $AlCl_3$ required for flocculation were $2000 \cdot 100 \cdot 1$.

Studies on chlorophyll–protein relationship have been made with

the aid of detergents, briefly by Kuhn *et al.* (122) and rather extensively by Smith (*loc. cit.*) and Smith and Pickels (*loc. cit.*). The primary visible effect of the detergents is to clarify the opalescent solutions. In the case of dodecyl sulfate, this also removes the Mg from the chlorophyll, converting it to pheophytin. Since, however, the chlorophyll-protein bonds remain, it is concluded that the complex is not bound through Mg. Pheophytin formation, which occurs in the above even at the pH range of 8–9, does not occur with digitonin or bile salts at pH 4–5.

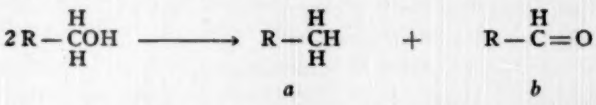
EFFECT OF RADIATION ON CHLOROPHYLL IN THE PLANT

There will be discussed in this section the effect of light on plants as a requirement for chlorophyll development, and destructive effects.

It is well known that light is required for the development of most, though not all, chloroplasts. Some algae, e.g., when fed a proper source of food, as glucose, can develop normally in the dark, producing chlorophyll as if they were normally growing in light. On illumination the capacity for photosynthesis generally does not appear to be inhibited. In the lotus embryo, Stewart and Eyster (218) report only chlorophyll *b*, with no *a* (see also Seybold and Egle (195) on pigments of spores and seeds). The majority of plants, however, require light not only for photosynthesis but for the very development of chlorophyll. The most direct method of investigation of the role of light is to determine its action spectrum, i.e., the effectiveness of the different wave lengths in producing chlorophyll. Using Corning filters in various combinations to give bands with a half-width averaging ca. 40 mμ, the effectiveness of the spectrum has been evaluated by Frank (67), using the amount of chlorophyll formation as a measure of the effectiveness. Under the conditions involved there was also carotenoid development, which was particularly prominent in the young *Avena* plants used and which affected the amount of incident light available to the plants. It was found, however, that the ratio of the effectiveness in chlorophyll formation of the blue light, which the carotenoids absorb, to that of red light, which they do not, did not change with age of the young plants (72, 96 and 120 hours old), despite a known doubling of the carotenoid concentration during that period. The conclusion was thus reached that the available light was not

screened out by the carotenoids, which must thus be located morphologically behind the light-absorbing active substance in the chloroplast. The action spectrum thus obtained (corrected for the spread of the filters) is given in Fig. 4. The spectrum resembles that for protochlorophyll relatively well. A partial explanation of the earlier difficulties may also be found in Seybold's (1939) (*loc. cit.*) reporting of the almost constant occurrence of protopheophytin along with protochlorophyll, also called "trichosanthin". The width of the band in the red, which does not occur to the extent found in porphines, must thus be attributed to an accompanying material; that is, at least two pigments are involved. This is made even more probable by the narrowness of the spectrum in the blue, where the bands of the two pigments would be expected to coincide. It is indeed possible that there are protochlorophylls corresponding to the separate chlorophylls *a* and *b*. Results consistent with the above have been given by Guerrini (88) with more monochromatic light. The contention of Barrenscheen *et al.* (16) that etiolated wheat seedlings do not possess protochlorophyll because their red-fluorescent ether extract did not possess a visible absorption spectrum, is unwarranted in view of the sensitivity of the fluorescence, which can determine porphyrin content 1/10 as much as the limit of visible absorption spectra. Ultraviolet light, however, poses a different picture, since protein may become denatured by its action. Although Eilert and Giersch (50) did not note any effect of the filtrate of UV-irradiated *Stichococcus* on the chlorophyll or carbohydrate content of algae (there is no indication of the extent of absorption by the algae of the materials), there is an abundance of evidence to show the debilitating action of the UV rays themselves. Thus Gilles (74) notes that, although plants containing chlorophyll are more resistant to UV rays than those not having it, since the chlorophyll obviously absorbs much of the radiation until it is itself destroyed and photooxidized, the wave lengths most effective in destruction are 290–310 mμ, where proteins as well as a variety of other substances are particularly effective absorbers. Montfort (146) emphasizes that the photooxidative destruction does not commence until photosynthesis has decreased to a small fraction of its nominal value. With Zöllner (148) he concludes that even strong light is not always harmful, as is the case in Nympheae.

Goodwin and Owens (80) report that on exposure to light (4358 Å) young oat plants form chlorophyll *a* but no chlorophyll *b* in three hours. The ratio of chlorophyll formation from protochlorophyll has been beautifully studied by J. H. C. Smith (214). It is shown that in etiolated barley seedlings the ratios of formation of chlorophyll *a* to *b* remain constant under a variety of temperatures and in both constant and intermittent illumination after a short period in which only chlorophyll *a* is produced. The suggestion is thus made, although it does not appear obvious, that chlorophyll *b* is not produced from *a*. The argument advanced is that if it were produced photochemically the ratio of formation would not be constant but continuously increasing. Since the discussion concerns ratios, not absolute amounts, it does not seem improbable that a definite fraction of the *a* is transformed photochemically to the *b*. That the ratio remains constant and similar during intermittent illumination as compared with constant illumination is the basis for indicating that *b* is not formed by *a* by a thermochemical reaction. This argument is faulty inasmuch as more *a* might well be produced in intermittent than in continuous illumination. If the amount of *b* formed is strictly a function of the amount of *a* present, then the above conditions do not invalidate the formation of *b* from *a*. The conclusion reached by the author and his associate is that chlorophylls *b* and *a* are formed from a common precursor by disproportionation, i.e.,



Other work by the same author (214) has shown that the amount of chlorophyll formed is directly proportional to the amount of protochlorophyll present in the leaf. Furthermore, by controlling the temperature and thus the rate of dark formation of protochlorophyll, he also controls the subsequent amount of chlorophyll formation. Biebel has reported (22), in work probably best interpreted as illustrating photoperiodism, that chlorophyll formation of kidney-bean seedlings increased with increasing frequency of light cycling up to 144 times per day.

The only recent report dealing with the effect of materials developed or used in radioactive investigation is that concerning the

effect of neutrons on photosynthesis in *Scenedesmus* and *Chlorella*. Nisina, Nakamura and Nakayama (158) irradiated these algae with fast neutrons from a deuteron (3 mev, 20 microamp.) bombardment of beryllium. A short irradiation of *Scenedesmus* (10′) had no effect on photosynthesis, while somewhat longer actually increased it, no inhibition being observed until after 50′ (36% diminution after two hours). In *Chlorella*, after one hour, a 46% diminution had occurred. Respiration in *Scenedesmus* was not affected by the 3-hour exposure.

CHLOROPHYLL IN RELATION TO PLANT COMPOSITION AND NUTRITION

Physiological studies often give determinations of chlorophyll along with those of numerous other constituents, e.g., potassium, nitrogen, iron, carbohydrates, ascorbic acid and organic acids. Occasionally conclusions are drawn regarding the relation between changes in the environment and their effect upon the isolated systems, as determined by analysis. It is obvious that in an organism in which the various portions are strictly dependent upon each other, anything which influences one will influence another. This is true not only in the larger sense but also in the smaller, i.e., with respect to the deficiency of a trace element, which affects, e.g., those of amino acid formation, will obviously affect protein formation. Disturbance of the protein will in turn affect all enzymatic reactions, since there are probably few if any of the latter which do not have a protein as a carrier.

It is possible, however, to gain somewhat of an insight into the relationship of reactions by a study of the rates when one of the factors is limiting. Plant physiological studies have not progressed in this direction sufficiently, however, and most problems have been attacked in a cause and effect manner. One of the better-known problems is that of the relationship between iron and chlorosis. Every student knows that the leaves of growing plants may be made chlorotic by a withholding of iron from the system. It has, therefore, been concluded that iron, per se, is involved in chlorophyll formation, and functions have been assigned to iron with evidence therefor (see section on theory of formation). Recent work on the nature of iron metabolism has been reviewed by Bennett (20). A variety of interesting relationships have been shown for

chlorotic pineapple (202, 204) fed iron and either ammonium ion or nitrate, not so much in the expected higher sugar yields but in variations of intermediates, as ascorbic and other organic acids. This would depend on the rate of nitrate reduction as compared to the rates of ascorbic formation as well as utilization. The ascorbic acid and other organic acids are decreased in the nitrate series; are they, therefore, involved in nitrate reduction? A similar but less ambitious study on sugar and N metabolism has been given by Cooke (39). More specific, however, is the work by Hill and Lehmann (97). These workers realize that the primary difficulty in analysis lies in the interpretation of the analytical data, i.e., of the various "classes" of iron resulting from different methods of determination. Thus there arose the concept of "active iron" by Oserkowsky (165). A linear relationship between chlorophyll and Fe was found when the active iron was plotted against the amount of chlorophyll. In this work the "active" iron was that extractable by dilute hydrochloric acid. Hill and Lehmann divide the water-soluble Fe into three categories: *a*) that which reacts with α, α'-dipridyl immediately; *b*) that which reacts only after boiling in acid; and *c*) that which reacts only after ashing. The Fe/chlorophyll ratio, varying from 1/4 to 1/10, also fluctuated with time, since the Fe in the chloroplasts preceded chlorophyll both in appearance and in disappearance. Jacobson (111) showed that if the leaves of the material were properly washed to remove surface iron, a proportionality could be found between total Fe and chlorophyll. However, before chlorophyll formation could occur, the iron in the chloroplasts had to exceed a threshold, indicating the prior necessity of other reactions for iron. A similar situation is known to exist for Mg. Thus, although a linear relationship was found to occur with the "active" iron and the chlorophyll, it also existed with other components, e.g., the protein precipitable by ammonium sulphate. Indeed the data are interpreted by Bennett (*loc. cit.*) as that of an association of the active iron with the active protein which is in turn responsible for chlorophyll formation. Thus it appears that the well-known iron-chlorosis phenomenon is more closely concerned with protein formation rather than chlorophyll formation. (See also 201 for analogous conclusions based on nitrogen studies.)

Studies in a similar light (e.g., 184) indicate that K "facilitates"

transfer of Fe and thus influences chlorophyll formation. The difficulties involved are further illustrated when careful workers as Sideris and Young (203) state that in pineapples, at least, chlorophyll or carotenoids are not affected by the K level of the cultures. Schropp (188) says that, although the effects of B on chlorophyll formation are variable, those of Cu, Mn and Zn definitely indicate chloroses in the event of deficiency. The necessity of these elements for various enzymatic reactions is becoming increasingly evident; e.g., witness the ability of Zn and Cu to relieve the inhibition by phenanthroline of the photochemical reduction of quinone by chloroplast grana (see photochemistry sections).

A direct approach to the effect of Mg deficiency has been given by Kennedy (115) in which the deficiency is studied not only as a function of the chlorophyll concentration but also as a function of the rate of photosynthesis per unit of chlorophyll per flash. It was shown that Mg-deficient *Chlorella,* in contrast to Fe-deficient, affected the rate of the "dark reaction", i.e., the amount of photosynthesis per unit flash was decreased if the dark period was not of sufficient length, which did not occur in Fe-deficiency. However, it is well known that Mg fed to deficient plants is localized in other compounds previous to chlorophyll [see, e.g., Smith (1947) in section on theory of formation and Jevillier and Goodchauz (112)], and it is therefore not possible to conclude from the above that chlorophyll has a dark function as well as a light function.

A host of papers exist on various factors and their relation to chlorophyll development or function. It is impossible to assess all, and a few are selected for illustrative purposes. A study has been made by Kohler (117), for example, of the composition of oats at various stages of growth. The factors analyzed included a number of the enzymes as well as fat, fiber, chlorophyll, protein, etc. The majority of the factors analyzed showed maximal amounts at the jointing stage. More or less routine determinations were made by other authors on pigment concentrations in fresh and processed Swiss chard and beet greens (176); on pigments in a variety of tobacco (86); and on assorted items, including pigments, of summer squashes (102) and of beet leaves (163). Soil studies include those (205) in which the effect of various types of fertilizers are

studied by noting the rate of photosynthesis of the plants, although it is difficult to understand why this is termed equivalent to the determination of chlorophyll, which would appear both easier and more convenient. A study has been made by Powers (177) of boron, which includes significant increase in chlorophyll (50%) after addition of B to soils deficient in that element.

Beck (19) studied the effect of drought on pigments of sunflowers, the previously dried soil being saturated with aqueous sugar solutions of various concentrations. It might possibly have been more advisable to regulate the water content with an inert carrier rather than introduce another variable. However, the major factors found to influence pigment production were not so much drought, this being effective only under extreme circumstances, but light and heat. Zaitseva (237) has found a temporary pigment effect after vernalization on wheat seedlings, the first leaves of vernalized seedlings showing more chlorophyll than non-vernalized seedlings.

Studies of the pigment content of algae continue. Graham (82) has published a generalized study of the chlorophyll content of plankton, showing that if due account is taken of the size and chlorophyll content of different species of plankton life, the chlorophyll content of plankton can be taken as an indication of the photosynthetic capacity of sea water. More specific studies for different groups of algae have been made by Seybold *et al.* (196). Skopintsev and Bruk (207) have studied the rate of decomposition of chlorophyll and phosphorus compounds of dead plankton. At 16° C., 82% of the chlorophyll is destroyed within 53 days, a condition similar to that for the P-compounds but much slower than that required for ammonification.

Spoehr and Milner (216), in describing the variation of *Chlorella* composition with differing inorganic media, noted that under conditions of low nitrogen the metabolism is shifted toward lipid synthesis. Comparison of cultures of increasing lipid content showed a decrease in chlorophyll content (down to 0.012% which is equivalent to 1/500–1/2000 of those of low lipid content), following which no further decrease occurred, though the cells continued to grow. The carotenoid decrease accompanying the chlorophyll change is only one-tenth the magnitude. The experiments are

noteworthy in their exemplification that the composition of a unicellular plant is not a constant, even within narrow limits, but can be made to vary with the environment. It is probable that the greater the number of cellular types (tissues) dependent upon each other, the less the allowable variation in composition. A similar study on NO_3^- and NH_4^+, less extensive and designed primarily for photosynthetic investigation, has been given by Myers (153).

The problem of the daily variation of the chlorophyll content continues. Using chromatographic technique, Wendel (230) made determinations of chlorophyll on three- to four-hour bases, finding a definite maximum at noon for four different plants. These variations were not found in darkened plants. Bukatsch (31), however, found the minimum weight of *Adenostyles* leaves to occur at the same time as their chlorophyll minimum, indicating a possible source of the variation. This is another of the type of problem which is vexatious and laborious by ordinary means and is readily solved with the use of isotopes, since all that needs to be done is to let a plant photosynthesize with isotopic CO_2 long enough to develop a certain amount of chlorophyll. Thereafter one need merely determine the specific activity of the chlorophyll at desired intervals.

An interesting genetic approach to chlorophyll formation, aside from that of the albinos previously mentioned, is that offered by Iuracec (110), in which it is noted that the chlorophyll content of fresh female and male plants of *Urtica dioica* are not equal but exist in the ratio of 1 : 1.15 (range 230–296 : 189–277). Obviously the first need is for more uniform tissue, following which cytological studies should be correlated with biochemistry. Miller and Johnson (142) believe that in F_1 crosses the genotype controlling chlorophyll concentration is formed by equal contributions from male and female parents. A continuation of the classical studies in modern form is given by Euvat (32) in which there are illustrations of the transformation of chloroplasts of mesophyll cells of *Brimeura amethystina* into amyloplasts during the time of petiole development. This is not, however, true of all chloroplasts, since those cells which have lost their somatic powers appear to be transformed into embryonic cells which can differentiate into new roots.

Two articles are available on chlorophyll concentration in poly-

ploids. Györffy (92) reports that in general the pigment content of polyploids is greater than that of diploids. Another distinction made is that the chlorophyll *a*/*b* ratio in diploids is usually greatest in the spring-summer period, while with tetraploids the greatest ratio occurs during the fall-winter period. In a more careful analysis, however, Pirschle (171) points out that, although tetraploids are richer than diploids per unit area, they actually contain somewhat less chlorophyll on a fresh-weight basis. The reason, of course, is that tetraploid leaves are usually thicker. The leaves of tetraploids are, however, generally larger, and per leaf tetraploids contain more chlorophyll. No difference was found in the normal pigment concentration ratios.

It is the opinion of the reviewer that one of the most fruitful approaches in plant physiology on the mechanism of tissue differentiation is that given by White (231) in which it is shown that a variety of perturbing forces may result in tissue differentiation, e.g., into leaves. It is, therefore, of interest to note that Loo (130) has been able to cultivate excised stem tips of dodder for five months through several transfers. Both flowers and lateral buds were produced without development of leaves or roots, although a large amount of chlorophyll was developed. The primary growth requirement appeared to be sugar, lyophylised whole cytoplasm not being as effective. It is, however, impossible to culture for an indefinite time leaves which cannot produce roots and thus become entire plants again. This is true regardless of the age of the leaves, so that one cannot regard the lack of success as primarily due to a natural limitation of growth via inhibition. Rather there must be some material formed by the root which sustains growth. This appears to be a most interesting field for study.

The relative amount of chlorophyll within leaves of a plant is not, however, a direct indication of the amount of photosynthesis that may occur, a fact long ago shown by Willstätter and Stoll (234). In a variety of circumstances it was pointed out that the amount of photosynthesis per unit of chlorophyll was often greater in leaves with less chlorophyll. This is, of course, readily explained on the basis that the photochemical reaction occurs only on the surface of the chloroplast and that the efficiency of energy transfer from within the chloroplast to its surface is not 100%.

Under these circumstances the relative efficiency of photosynthesis would increase with decreasing chlorophyll content down to the region of depopulation of the chloroplast surface. Shcheglova (199) has shown another technical variation in buckwheat leaves where the amount of photosynthesis in early summer is greater with leaves containing a small amount of chlorophyll, the reverse being true in late summer. The effect, explained by her on the basis of a temperature effect, is also just as readily explained as a light effect, if the light is not saturating in early summer but is in late. Roubaix and Lazer (185) find no relation between chlorophyll content in leaves and sugar in the beet. The compensation points of a variety of sun- and shade-loving plants have been given (186). Comparisons have been made over a three-year period on chlorophyll, carbohydrates and water content of bean leaves grown in light and in partial shade (109). The chlorophyll ratio (sun to shade) was 1.3 on a fresh-weight basis, but 0.99 on a dry-weight. The corresponding average CO_2 assimilations was 1.14 and 0.82. The respiration is greater in the sun leaves. Emerson, Green and Webb (52) have pointed out in a more quantitative fashion that the maximum amount of photosynthesis per flash of light is not directly related to the amount of chlorophyll so much as it is to internal factors, e.g., previous conditions of growth (of algae), color and intensity of illumination, and age of culture. Pickett and Kenworthy (170) believe that in apple leaves the amount of "initially" exposed surface is more important for photosynthesis than the absolute amount of chlorophyll.

The amount of chlorophyll at various times in the life history of a plant, or under varying conditions, have long been popular studies; they continue to be. Zaitseva (238), for one, notes the increase in chlorophyll content of wheat leaves from vernalized winter wheat up to the point where the last leaf no longer envelopes the spike, after which the chlorophyll content begins to decrease. The decrease was not apparent in non-vernalized wheat. In rice, barley and wheat, Nagasima (154) reports two maxima, one during most active growth and the other during the reproductive season. The latter maxima is not a certainty. Olsen (164) finds only a single long-period maximum (June to autumn) for beech leaves. As in Iuracec's work (*loc. cit.*) chlorophyll content per unit surface is higher, though it is equal on a dry-weight basis, for leaves exposed to sun as compared to those in shade.

PATHOLOGICAL ASPECTS

The characteristic pattern produced is the simplest means of recognizing chlorophyll destruction caused by tobacco mosaic virus and has induced an interest in the mechanism of chlorophyll degradation. Dufrenoy (46) relates this to enhanced oxidase activity during virus infection. The increase in enzymatic action is presumably due to increased substrate arising from the degradations of natural proteins, etc., due to the virus. Because of the autocatalytic nature of the infection this enhancement never diminishes, and the chlorophyll is thus destroyed through oxidase activity (see section on enzymes). Actually we have no knowledge of the mechanism of chlorophyll degradation beyond that of the chlorophyllides, due to chlorophyllase. We have no indication whether degradation proceeds via oxidation or reduction and have not the slightest hint as to the nature of the lenco compounds. In contrast to Dufrenoy, Gol'din (79) presents data which indicate little difference in chlorophyll content between sound and affected plants, the weight, if any, being in favor of the affected leaves. He believes that the rate of formation of the virus does not always parallel the delay in development of the chloroplasts. At variance with both opinions, McKinney (133) states that not only did virus-infected tobacco have less chlorophyll (30% less) as well as a decreased chlorophyllase activity, but also had decreased oxidase activity. In cotton with the virus producing leaf curling, Kar-Murza (113) reports less chlorophyll in infected than in healthy leaves. It is obvious that in the same plants with the same virus, the same results should be obtained. It is of course conceivable, however, that the virus in different strains acts either more slowly or in a different fashion, so that chloroplasts are affected either at different rates or so indirectly that the chloroplasts, which are to a certain extent independent, may not seem to be affected at all.

In fungus diseases it is possible, as Müller (152) has pointed out, that an organism can in one instance act as a symbiont, as *Marasmius oreades* on *A. millefolium,* even though chlorophyll content is reduced, but as a parasite on others.

Needless to say, the use of certain sprays or control chemicals, e.g., naphthalene acetic acid or Pb-arsenate (217), which affect the normal development of cells, may well be expected to affect the amount of chlorophyll development. It is interesting to note, however, that in the former, although chlorophyll is diminished, ascorbic

acid is not. Pickett and Birkeland (169) believe that some of the lack of cell development of Pb-arsenate-sprayed leaves may be due merely to unavailability of surface layers of the palisade cells for normal reactions, not to a specific chemical effect.

ENZYMES IN CHLOROPLASTS

Included in this section are several papers, not necessarily related, which have in common the biochemical approach as well as the common spatial environment of the enzymatics involved.

Of primary interest, especially in view of the demonstrated ability of the photochemical reduction of quinones by chloroplast grana (10, 226–228), is the relationship between the naphthoquinone, vitamin K, and chlorophyll. Dam, Glavind and Nielsen (40) have shown that the chloroplasts of cabbage have 60 times as much vitamin K as the cytoplasm. Furthermore the vitamin K concentration is proportional to the chlorophyll concentration which in turn is generally proportional in young seedlings to the amount of light. It would be interesting to know whether light itself is really required for vitamin K formation as it usually is for chlorophyll[4]. A similar relationship has been reported for ascorbic acid, although here too the direct relationship is still in doubt. Continuing in this problem is the work of Neubauer (156), in which this relationship is reiterated and carotenoids are also mentioned in the same respect. It is of course well known that carotenoids always accompany chlorophylls, though the reverse is not by any means true.

Another parallelism which exists is that of catalase–chlorophyll (45, 166). Nakamura (155) and Kashimoto (114), continuing previous suggestions of the former, found that most plant catalase is in the chloroplasts in higher plants, and that in the lower as well as the higher the chlorophyll–catalase relationship existed. Similarly in chlorotic and aged yellowed leaves both have decreased. It is, however, well known that many if not all porphyrin compounds have catalase-like powers. It is interesting to note, therefore, that catalase itself actually exists in these tissues (114) and

[4] One may thus speculate that if the process of photosynthesis is merely the reverse of respiration (33) no basis is afforded for unique syntheses, since such reversals may occur, though to a lesser extent, in respiration itself. Either the compound(s) or its intermediate(s) in photosynthesis is in some manner different from those in respiration, or other unique photochemical reactions occur apart from photosynthesis as it is usually understood.

that chlorophyll is not performing the function assigned to catalase. Krossing (121) has described a method whereby green leaves are divided into four portions: chloroplasts, cytoplasm, water-soluble portion, and cell wall material. A study of the distribution of various enzymes showed that the chloroplasts alone contained the chlorophyllase and amylase, and contained the predominant amount of catalase, while the sole substance identified confined to the water-soluble fraction was the peroxidase; the cytoplasm also had significant amounts of catalase, both it and the chloroplasts possessing nominal amounts of sucrose. Sisakyan and Kobyakova (206) find invertase, amylase and protease in plastids. Yin and Tung (236) have provided a histochemical demonstration of the localization of phosphorylase in the guard cells of epidermal tissue.

Pallares *et al.* (167) have discovered a most interesting relation in amphitrophic *Euglena viridis*. In a medium containing glucose, cellobiose and lactose, and in media to which various vitamins were added singly (A, D, E, K—synthetic—, B_1, B_2, B_6, C, H, P, PP, pantothenic acid and inositol) and then kept in the dark for two weeks all cultures except those containing vitamin H (biotin) (1/100 ml.) and vitamin C (ascorbic acid) (200 mg./100 ml.) lost their chlorophyll. Assuming that the effect is not due to that of minor element contamination (e.g., Fe, Cu), the effect and the organism could be used as a means of tracing in part the mechanism of chlorophyll development. Elliott (51) notes an effect of growth stimulation on *Euglena* in light but not in dark with phytohormone. In this regard the work of Gustafson (91) is pertinent. He reports that corn seedlings, devoid of chlorophyll either because of being grown in the dark or because of genetic constitution, had more growth hormone than normal similar plants. The relationship is thus still obscure unless this is merely an instance of photosensitized oxidations. A possible cytogenetic effect of saponins is indicated in the work of Balansard and Pellissier (14). Algae (Protococcae) grown in saponin (quillaja and polygala) solutions (10^{-4}–10^{-5}) formed more chlorophyll than the controls. Furthermore ivy with mottled leaves put out new leaves which were entirely green.

Arnon (4, 5) has shown that chloroplast grana are the seat of the polyphenol oxidases in green leaves (chard).

Von Euler (56, 57) has noted in a series of papers that addition

of streptomycin to germinating seeds results in a lack of chlorophyll formation. The mechanism is believed to involve disruption of nuclear functioning, especially of nucleic acids.

Among the more interesting of the enzymes present in many plants is that which hydrolyzes phytol from chlorophyll, i.e., chlorophyllase. In a study of this enzyme (229), which is active at room temperature in such strange solvents as 80% ethanol and 40–70% aqueous acetone as well as in water at 75°, the authors point out the variability both with single plants and among different plants as well as its difficulty of extraction.

Noack (160), continuing his previous studies on chlorophyllase, believes that it functions primarily in the decomposition of chlorophyll, since once the water-soluble chlorophyllide is formed, it can be more or less readily oxidized by peroxide. The conditions for this oxidation are believed to occur in conjunction with iron as colloidal ferric hydroxide. Indeed it is shown that it is possible to oxidize chlorophyllide completely to carbon dioxide, ammonia and water, with hydrogen peroxide and ferric hydroxide. The optimal conditions in a leaf at which this destruction may occur are those just before the leaves turn yellow (*sic!*), when catalase activity is diminished.

Boichenko (25) found that chloroplasts possess a hydrogenase system which could be activated by methylene blue. Both oxygen and carbon dioxide could be reduced by molecular hydrogen (the latter goes to formate even in the absence of oxygen). The reactions are reversible, and gaseous hydrogen may be produced for an extensive period.

JOINT ACTIVITY OF PIGMENTS

By means of action spectra it has been known since Englemann's time that in red algae containing phycoerythrin, a water-soluble bilin protein, that pigment is capable of taking part in the light reaction in photosynthesis. This is of extreme interest, indicating either that it is not an absolute prerequisite that chlorophyll take part in photosynthesis, or that a coupled reaction of some type occurs between the pigment systems, one of which is in the cytoplasm and the other in the chloroplasts. Haxo and Blinks (23, 70*a*) have recently shown that in the red algae, which contain in their chloroplasts the water-soluble bilin-proteins, phycoerythrin

and phycocyanin, the more phycocyanin the less operative is the chlorophyll. In the genus *Porphyra*, containing the most phycocyanin, the chlorophyll is essentially inactive. Although under abnormal conditions where the phycoerythrin is leached out by the sun, the chlorophyll is again operative.

This is, therefore, the first authenticated instance of a genus containing chlorophyll which is non-functional. The authors give no explanation for the inactivity of the chlorophyll; indeed, the assumption had heretofore been made that these water-soluble pigments operated through the chlorophyll by transferring energy to them. Mathematically formal analogies in comparing this energy transfer to the process of internal conversion within the atom emitting gamma rays have been proposed (3*a*).

This analogy, while possibly of interest in relation to the similar problem of sensitized carotenoid transfer, appears, therefore, irrelevant when applied to the bilins. These pigments are, of course, closely related to the porphyrins, and there is no reason why they should not be capable of participating in the photochemical reaction *per se*. It is even possible that in a competition for the limited hydrogen acceptor the bilins should be superior to the chlorophyll because of their water-solubility, although this will not of itself explain the poor yield of chlorophyll action and still less its inactivity. The fact that after leaching of phycocyanin the chlorophyll is again functional, and that in other genera with lesser amounts of phycocyanin the chlorophyll is at least partially active, implies an actual inhibition of chlorophyll activity by phycocanin, possibly by competition for the catalytic sites on the protein surface. Increasing evidence indicates that within the chloroplast itself, other pigments, e.g., the carotenoids, may take part in photosynthesis. This point of view has been maintained qualitatively for some time by Montfort (145, 147). Quantitatively this has been presumably demonstrated (49) in the diatom *Nitzschia closterium*, in which, at those wave lengths where absorption of carotenoids is considerable and of chlorophylls minimal, ascribing the entire photosynthetic yield to the chlorophylls would imply an efficiency greater than 100%. A similar result was obtained for the alga *Chroococcus* (53). The manner of transfer of energy from the excited carotenoid to chlorophyll has not been postulated. No addition

compounds are formed between chlorophyll and carotenes, since absorption spectra of mixtures are strictly additive (12). However, in the highly concentrated states existing within the cell, very strong interactions are not only possible but probable. There remain at least two additional explanations for this apparent relationship, both of which have been anticipated to some extent (48). First it is not at all impossible that the extinction coefficients of the pigments within the leaf do not correspond with the extracted material, so that proper assignment of relative absorption of light has not been made.

This difference may arise from either of two sources: *a*) association with other substances or with itself, or *b*) orientation of the chlorophyll so that it is not randomly distributed with respect to the vector of incident light. In the work of Dutton *et al.* (*loc. cit.*) and Dutton and Manning (*loc. cit.*), a complete negation of the effect of carotenoids would involve an increase in the extinction coefficient at 5,000 Å of 2–3-fold. This does not appear likely, nor are the differences of vitro shifts of absorption spectra, compared to those of extracted material, likely to account for differences of such a magnitude. A second alternative, foreseen though not discussed by these workers and suggested for *Avena* seedlings by Frank (67), is the spatial separation of the pigments within the chloroplast. It was found that despite large increases of carotenoids with time (double in the period of 72–120 hours), the effectiveness of the incident (blue) light for chlorophyll formation was unimpaired. These results were regarded as showing not only that the carotenoids did not screen out the light by being in front of the protochlorophyll and were not in statistically equivalent positions, but that they were actually situated behind the protochlorophyll. It is not impossible that the reverse is true in *Nitzschia*, at least for the carotenoid involved. Indeed, that some carotenoids screen, and others do not, may explain why some appear to be active, and others not. The conception appears, however, to be probable only in the case of a monolayer. Indeed a similar explanation for the effect could be ascribed to an adjacent disposition of the carotenoids. Either theory would appear to be precluded in case of multilayers or multicellular tissue unless there is a precision of alignment within the chloroplasts and of the chloroplasts within

cells, of which we are not yet aware. A simpler explanation is that the light absorbed by the carotenoids is transferable to the protoporphine for chlorophyll formation.

OPTICAL ACTIVITY

Use of the optical activity of chlorophyll derivatives, which once played an important, though misleading, role in the determination of chlorophyll structure (see, e.g., the review by Fischer, in Chem. Rev. 1938), has had revived interest, especially in the determination of the degradation products of the oxidation of chlorophyll derivatives. Furthermore reports have occasionally been made of the inactivity of compounds which were expected to be active, with reappearance of activity on further transformations. Pruckner, Ostreicher and Fischer (179) have shown that a number of these anomalies are due to the effect of the wave length of the light used to take the measurement (because of the pronounced change in dispersion near an absorption band). It is, of course, an unfortunate habit that has arisen, of reporting optical activities at only one wave length, inasmuch as these are of no more value than if absorption spectra were given at but a single wave length. The effects of concentration are also given, the results of which may indicate varying degrees of association. In using a red filter (690–720 mμ), for instance, the following values are listed for methyl chlorophyllide *a*:

Mg. / 100 cc. acetone	$(\alpha)^{20°}$
10.65	660
5.37	940
2.68	740

The results with mesopheophorbide are even more anomalous.

REVERSIBLE BLEACHING

Porret and Rabinowitch (175) found that in oxygen-free solutions (methanol) chlorophyll is reversibly bleached with strong light (ca. 1% with 2000-watt carbon arc, with a red filter). The effect was increased markedly with formic acid. The intensity of the light was such that for the concentration of chlorophyll employed, each molecule absorbed a quantum of light once a second; the color returned in the dark in about ten seconds. Livingston (125, 126) repeated the experiments, using more purified solutions

and weaker light. Although the regeneration time was found to be much increased as a result of the purification of solvent (to 100 seconds), the stationary bleaching was confirmed to be proportional to the square root of the light intensity. This was interpreted by Rabinowitch[5] as a bleaching effect, proportional to the light intensity, and as a regenerating effect, proportional to the square root of the regenerating bleached product. The assumptions of Porret and Rabinowitch in this regard were confirmed by direct determination of the regeneration time in Livingston's work. Rabinowitch (*loc. cit.*) lists four possible mechanisms for chlorophyll bleaching: tautomerization, dismutation, oxidation, and reduction. The first should result in a monomolecular back reaction, which is not the case; the second is excluded on the basis of concentration (ca. 10^{-5} *M*), although this possibility is not entirely ruled out. McBrady and Livingston (132) propose a combination of tautomerism and dismutation:

$$\begin{aligned}
GH + lite &\longrightarrow GH^{*} \quad \text{(absorption)}\\
GH^{*} &\longrightarrow GH + lite \quad \text{(fluorescence)}\\
GH^{*} &\longrightarrow HG \quad \text{(internal conversion accompanied by tautomerism)}\\
HG &\longrightarrow GH \quad \text{(spontaneous reversion)}\\
HG + GH &\longrightarrow GH_2 + G \quad \text{(dismutation)}\\
GH_2 + G &\longrightarrow 2\,GH \quad \text{(regeneration)}
\end{aligned}$$

The possibility of reversible oxidation is minimized by the probability of the oxidation of the reduced product by oxygen, which inhibits the reaction, leaving oxidized chlorophyll. The effect of oxygen could then be explained only by a catalytic acceleration of the reaction.

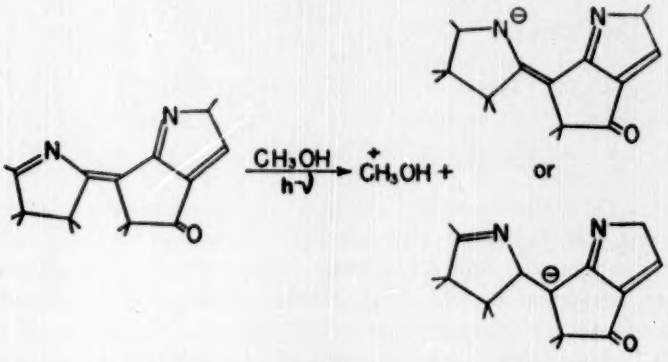
The possibility of a triplet state for the excited state of chlorophyll *b* has been suggested by Calvin and Dorough (35). Since chlorophyll *a* has not yet been shown to exhibit such phosphorescence the nature of the excited state is still in doubt. Were such a state to exist, then, as Livingston suggests, the inhibiting effect of oxygen would be due to its paramagnetism, the field effects of which would diminish the lifetime of the excited state of the chlorophyll. Reduction, however, is another possibility, since this would

[5] For a thorough discussion up to the time of publication (1945), see Rabinowitch, "Photosynthesis", vol. 1, Interscience Publishers, N. Y.

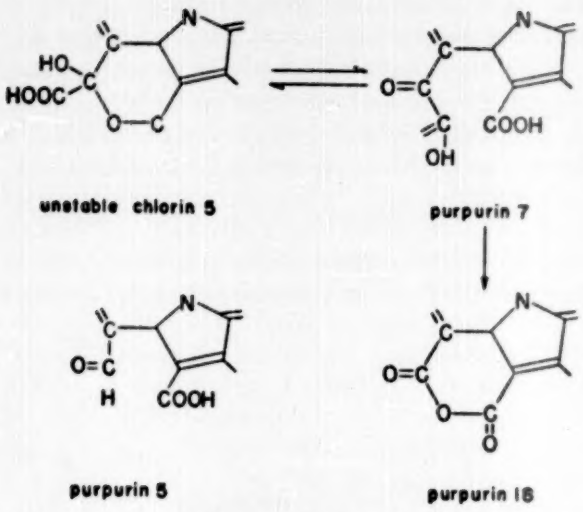
leave only oxidized solvent in the presence of oxygen. Rabinowitch considers the possibility of the reaction of chlorophyll with solvent: Chph + S ⇌ r Chph + oS, to be an important alternative to consider. Indeed one can use the colorless triphenyl carbinols in methanol as analogues (99). The action of light in these studies was to eject an electron to form a colored compound:

$$CH_3OH + C_{31}H_{24}N_2 \longrightarrow C_{31}H_{25}N_2^+ + CH_3O^-.$$

The analogous reversed reaction could be conceived of as occurring at the δ-carbon or IV-nitrogen, resulting in loss of conjugation:



There is also the lesser possibility that the reaction may not be due to chlorophyll itself but to an equilibrium between some of its degradation products. More specifically, intermediates of the phase test, or allomerized material (both of which Rabinowitch indicates may be involved) give a direct example of the possibilities. Consider the phase test sequence: unstable chlorin 5 to purpurin 7 to purpurin 18; the corresponding absorption coefficients at the red maximum are $4.5 \cdot 10^4$, $3.0 \cdot 10^4$, and $5.8 \cdot 10^4$. If the action of light is involved in the equilibrium between the unstable chlorin and purpurin 7, then neither oxidation nor reduction is involved, though a large decrease in absorption coefficient occurs. It is interesting to note that the absorption coefficient of purpurin 5 at its maximum in the red is $4.5 \cdot 10^4$. The explanation of the reduction of the absorption coefficient by purpurin 7 would thus not be directly traceable to the alpha-keto group:



In this regard the work of Calvin and Dorough (*loc. cit.*) is of interest. It was shown that a typical synthetic chlorin (Zn salt) could be photooxidized by quinone to the corresponding porphin. Here, too, traces of oxygen inhibit the reaction, and the explanation offered by Livingston for the oxygen effect is also employed here. Since it was found that the rate of the reaction was independent of quinone, down to very low concentrations, it was believed that the quinone did not compete with those mechanisms which deactivate the first electronic state (fluorescence, solvent, wall, etc.), but reacted with some other state, e.g., the triplet, obtainable from the excited state. The Zn chlorin was shown to be phosphorescent and possess a triplet-state lifetime of approximately 8×10^{-3} sec. Calculations showed that the time of active collisions between the chlorin and the quinone at the limiting concentration of the latter would be made in 10^{-5} sec. It was thus concluded that each triplet state molecule could react with the quinone until the concentration of the latter became so low that other deactivating processes occur. The Cu chlorin, which is not phosphorescent (or, if phosphorescent, of very short life-time) was also non-reactive photochemically.

Those experiments are of interest to chlorophyll studies, since there is no a priori reason why it should not act in a similar

manner. Unfortunately chlorophyll *a* does not phosphoresce with a reasonably long life time, and probably some other type of longevity of an excited state, rather than the triplet, must be postulated [in this regard see the discussion by Franck and Livingston (66)]. It is of interest, however, that quinone can be used to effect the photodissociation of water, using chlorophyll grana, and under these circumstances the chlorophyll could well act as an H-carrier, e.g.,

$$\begin{aligned}
\text{Chph} + h\nu &\longrightarrow \text{H} + \text{"Photochph"} \\
\text{"Photochph"} + H_2O &\longrightarrow \text{Chph} \cdot \text{O (or Chph} \cdot \text{OH)} \\
2\ \text{Chph} \cdot \text{O} &\longrightarrow 2\ \text{Chph} + O_2 \\
\text{or } 2\ \text{Chph} \cdot \text{OH} &\longrightarrow 2\ \text{Chph} + 2\ \text{OH} \cdot \\
2\ \text{OH} \cdot \longrightarrow H_2O_2 &\longrightarrow H_2O + \tfrac{1}{2}\ O_2
\end{aligned}$$

Norris *et al.* (162) attempted by use of tritium to demonstrate the ability of chlorophyll to act as a hydrogen carrier, and were unsuccessful. Tritium is subject to difficulties both because of its large mass and because of attendant slow diffusion. Furthermore equilibrium constants involving tritium may be considerably less favorable than hydrogen. These authors suggested that use of 100% deutrium oxide could solve the problem unambiguously. Unfortunately this is not the case. Experiments with heavy water, and in conjunction with C^{14} (34), showed that, although there was more deuterium in the chlorophyll from algae exposed to light than in those kept in the dark, the amount of deuterium found was only ¼ of that expected. Furthermore the increased deuterium in the chlorophyll from the algae in light could be accounted for by increased synthesis (growth) in the light. These preliminary experiments indicate that chlorophyll may act as a sensitizer rather than as a hydrogen transport agent, or that, if it does act as a hydrogen carrier, *a*) it functions via an enolizable hydrogen, or *b*) the fraction of chlorophyll that is prominently active is small compared to the total amount, or *c*) the path of hydrogen from water to chlorophyll proceeds via a large reservoir of non-exchangeable hydrogen.

The reader is referred to Rabinowitch (*loc. cit.*) for an excellent discussion of earlier papers concerned with various photochemical studies on chlorophyll and significance of the apparently reversible bleaching of chlorophyll by Fe^{+3}. Of unique interest among these earlier studies is that by Gaffron (71) on the reversible photooxi-

dation of isoamylamine with chlorophyll. In a typical experiment, in which 1 cc. of isoamylamine was added to an acetone solution containing 1 mg. of chlorophyll, with an atmosphere of oxygen, illumination of the system caused absorption of 288 cmm. of O_2 in 5'. 95% of this oxygen could be released by addition of small amounts of MnO_2. Thus the chlorophyll sensitizes the photoformation of a moloxide. If water was present, H_2O_2 was formed. Addition of catalase to such a system would thus result in the continuous decomposition of water, analogous to the important type reactions involved in photochemical reduction.

PHOTOCHEMICAL REDUCTION BY CHLOROPLASTS AND GRANA

Englemann (54) showed, by means of bacteria motile only in the presence of oxygen, that the oxygen evolved during photosynthesis arose from the chloroplasts. That the gas thus evolved was oxygen was later shown by means of the color change of hemoglobin to oxyhemoglobin. Ewart (55) extended the earlier observation to isolated chloroplasts, in which, however, a greatly diminished activity, ceasing after a few hours, was noted. The experiments were in part duplicated and confirmed by Beyerinck (21), using luminescent bacteria which specifically require oxygen for luminescence, although only in small concentration, and the intensity of which may, to a certain extent, be used as a quantitative index of the amount of photosynthesis. More recently, using a phosphorescent dye quenched by oxygen, Franck (65) has measured the amount of oxygen evolved by chloroplasts quantitatively. The amounts of oxygen in these experiments were, however, of so minute an amount that ordinary Warburg manometric technique, in which one is concerned with ca. 10^{-6} mole of gas, was valueless.

It was thus of considerable interest that Hill (95) was able to show the evolution by chloroplasts, in light, of oxygen in the presence of ferric oxalate. The phenomenon was interpreted as a photolysis of water:

$$2\,Fe^{+3} + 2\,HOH \longrightarrow 2\,Fe^{+2} + 2\,H^{+} + O_2,$$

in which chlorophyll had served as a catalytic agent, and in which, because of the wave length of light used to effect the photodecomposition, energy had been transformed from electromagnetic to chemical, as in photosynthesis. Later, with Scarisbrick (98), the

conditions of the reaction were extended so that, e.g., the rate of reoxidation of Fe^{+2} by oxygen, which limited the accumulation of the gas, was retarded by the use of ferricyanide. By the use of a variety of substances which normally poison or inhibit photosynthesis, azide, cyanide and fluoride, only the urethanes, which narcotize the reaction, were shown to have an inhibiting effect. Later work (e.g., 6, 103) conflicts on the inhibiting effect of azide. Although Aronoff obtained results in agreement with Hill, Arnon explains the lack of inhibition as due to the absence or low concentration of halide. This poses an interesting question as to why the reaction proceeds uninhibited by azide or hydroxylamine at low halide concentrations, albeit at lower values than if halide is present. The reaction was believed to be identical with that occurring during the light reaction in photosynthesis.

The sensitized photolysis of water is well known, e.g., that of ZnO or Ag_2O with UV, although, because of the wave lengths of the ultraviolet light used, energy is not stored. The reader is referred to the discussion of this and further examples, as mercury-sensitized decomposition of HOH, in Rabinowitch (*loc. cit.*).

Hill's work was extended in this country by that of French and co-workers (69, 70, 103–105, 123), who not only established the production of hydrogen ion in the reaction but further defined optimal conditions, such as pH, chlorophyll concentration and quantum yields, and gave proof of the source of the oxygen as being the water rather than the oxidant.

Further advances in this regard were made independently by Warburg and Lüttgens (226) and by Gurevitch (89, 90), who were able to show the photoreduction of organic compounds by chloroplasts in light, the latter having effected the reduction of dinitrobenzene to nitroaniline, and the former of quinone to hydroquinone. Warburg and Lüttgens, especially, showed the catalytic nature of the reaction, ten times as much oxygen having been evolved as there was chlorophyll present, and emphasized the energy storage of the process, as in photosynthesis. A later communication (227) showed that the reaction could also be obtained if the chloroplasts were broken down into smaller fragments, called grana, precipitable in nominal time by centrifugation only at relatively high speeds (ca. 20,000 g). Activity was shown to disappear by dialysis of the grana, and could be reinstated only on

addition of minute amounts of halide (except fluoride) or nitrate ion. Arnon has recently shown (7) that the halide ion, not essential for plant growth, in some manner protects the photochemical system from the destructive effects of light. Furthermore the process was shown by Warburg and Lüttgens to be capable of poisoning with phenanthroline which was stated as being capable of removal and consequent reactivation of the reaction merely by washing. In their more extensive exposition (6) this was shown to be the remarkable effect of Zn which was more effective than excess equimolar amounts of iron. Arnon has also shown (6) that Cu is at least as effective as Zn, and that Co and Ni are more so. Both Warburg and, independently, Aronoff (10) showed that algae could evolve O_2 in the absence of external carbon dioxide. Fan, Stauffer and Umbreit (60) had previously shown that algae could reduce benzaldehyde and evolve oxygen thereby in the presence of light. Aronoff reported further the ability of other quinones, e.g., naphtha- and anthraquinones, to be used as oxidants. Correlations were made at different wave lengths with redox potential of the quinone-hydroquinone system, relationships existing in strong light being reversed in weak.

Gurevitch (*loc. cit.*), who demonstrated photoreduction by isolated chloroplasts of *o*-dinitrobenzene to *o*-nitrophenylhydroxylamine, showed that in the intact leaf the reduction proceeds to a further extent, namely to *o*-nitroaniline. The difference is presumed to indicate a loss of reduction potential on the part of the chloroplasts, due to their preparation, rather than loss of cytoplasmic factors which could effect such a reaction.

Boichenko (25, 26) has reported not only the oxidation of leuco dyes by chloroplasts but their assimilation of carbon dioxide, as evidenced by changes in the pH of the medium. This has not been confirmed (Aronoff, *loc. cit.*) and could not be shown [(29), Aronoff, 1947, unpublished] by means of $C^{14}O_2$. Any CO_2 uptake in Boichenko's experiments must thus be the result of a hydrogen-ion effect due to light (e.g., a photodecarboxylation) or a time change in the system. It is interesting to note that Warburg and Lüttgens' original contributions were entitled "Carbon Dioxide Assimilation", but no evidence was given of the inference.

Among the most important of the photochemical reductions is that in which an actual gain in free energy has been reported. Krasnovskii (120) reports photochemical reduction of riboflavin

($E_{pH=7} = -0.22$ volts) by ascorbic acid ($E_{pH=7} = +0.05$ v) in the presence of chlorophyll or Mg phthalocyanin. The reaction is reversible in the dark. There is no reason to believe that ascorbic acid, which is present almost solely in the chloroplasts and only so long as chlorophyll is present, does not perform a similar function in photosynthesis. In fact one may depict the beginning of a system of those substances known to be localized in the chloroplast and which may be involved in the photochemical reaction:

$$R^* + \text{ascorbic acid}\dagger \xrightarrow[\text{light}]{\text{chlorophyll}} RH_2 + \text{dehydroascorbic}$$

$$[2\ RH_2 + CO_2 \longrightarrow (CH_2O) + HOH + 2\ R]$$

$$\text{dehydroascorbic} + \xrightarrow[\text{or naphthoquinols}]{\text{polyphenols}} \text{ascorbic} + \text{quinones}$$

$$\text{quinones} + HOH \xrightarrow[\text{light}]{\text{chlorophyll}} \text{phenols} + O_2$$

In any event this reaction will probably play an important role in elucidation of the photochemical mechanism in photosynthesis.

Occasionally reports have come of photochemical reductions involving synthetic states of chlorophyll [e.g., (17)]. A solution of chlorophyll in geraniol or phytol emulsified with glycerol containing some methylene blue was given an atmosphere of CO_2 and illuminated with a strong tungsten lamp, resulting in assimilation of some of the carbon dioxide and evolution of formaldehyde and oxygen. The present writer (1945, unpublished), repeating the experiments with ordinary Warburg technique, found only a strong photooxidation and utilization of oxygen instead of its evolution, but these experiments were of the static type, not of the flow methods used by the above authors. Zhukovskii (240) found that in using "strong" infra-red (presumably in the neighborhood of 3.5 μ, where CO_2 is a strong absorber), formaldehyde is produced by reaction of carbon dioxide with water. Inasmuch as this reaction requires a $\Delta F = + \sim 125$ kcal., this would require approximately 15 quanta, with light of the above-mentioned wave length,

* R is the cytoplasmic hydrogen acceptor (e.g., as Krasnovskii suggests, DPN or TPN).

† It is possible that a compound as aldopyruvic acid, which could be a photosynthetic intermediate, may be operative here also. Indeed, ascorbic acid may be formed in vivo from condensation of aldopyruvic acid and glyceraldehyde. The unique formation of ascorbic acid in the green plant tissues would thus be explained.

an extraordinarily inefficient reaction compared to known values for photosynthesis. Furthermore the photochemical reaction nominally implies an activation to an excited electronic state, in which higher frequencies would be involved instead of the vibrational-rotational level of this infra-red.

It is, however, established that a material may be prepared from leaves which does not contain any visible cell structure and which, though usable only as a colloidal suspension, appears to duplicate the photochemical reaction occurring in photosynthesis. It thus becomes possible to test various theories with regard to the function of chlorophyll in photosynthesis, with due regard to "foreign" substances still absorbed on the surface despite care of preparation, and to enzymes present but not taking part in the photochemical reaction. Examples of these questions which may be tested are *a*) whether chlorophyll acts as a hydrogen carrier or merely as a photosensitizer, *b*) whether phosphorus is involved in the photochemical reaction, and *c*) whether the chloroplasts are involved to any extent in carbon dioxide assimilation or merely serve as a source of reducing power.

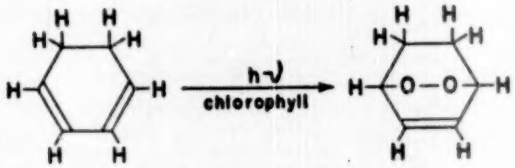
In other words the isolation of the cell fragments may serve as the first steps permitting elucidation of the biochemistry of the photochemical reaction in photosynthesis.

PHOTOOXIDATIONS

The ability of chlorophyll to act as a photosensitizer is known primarily because of its action as a photooxidizing agent. Examples of the purposeful use of chlorophyll in this manner are the following:

O. Richter (182) made use of chlorophyll solutions as sensitizers for gypsum photographic plates.

Schenk and Ziegler (187) have used chlorophyll solutions to effect peroxide formation in a number of analogous reactions of the type:



The quantum efficiencies of this reaction are quite high ($\gamma = 1$). Yamahuzi *et al.* (235) have demonstrated the photochemical production of H_2O_2 from aqueous acetone solutions in the presence of a sensitizer (chlorophyll) and a small amount of oxidizable organic matter (turpentine).

It is, of course, well known that chlorophyll itself is decomposed by light in the presence of oxygen, although it is quite stable in the absence of oxygen or solvents which do not contain peroxides. Aronoff and Mackinney (12) have studied the rates of photooxidation of purified chlorophylls *a* and *b* in benzene, using monochromatic light from a mercury arc (4358 Å). The reactions in both instances appeared to be of the second order and approximately equal, in contrast to the differing rates of pheophytin formation. The quantum efficiencies were very low, being of the order of 10^{-5}. Various substances, as carotene, afforded a certain amount of protection. In the presence of oxygen it could be shown that whereas some substances, as ascorbic acid, are photooxidized by chlorophyll, others are actually protected by the chlorophyll because of its absorption of near UV. Pepkowitz (168), using crude chlorophyll and non-monochromatic light, has conducted similar studies, primarily with respect to photochemical destruction of carotene.

Krasnovskii (119), previous to his classic experiment on photochemical reduction of riboflavin by ascorbate, showed that in the absence of oxygen, either ascorbic acid or phenylhydrazine causes loss of fluorescence and color, which can return in the dark. The chlorophyll, however, is altered, having lowered absorption coefficients both in the blue and, in the case of chlorophyll *b*, in the red.

Mention has previously been made of the sensitized photooxidation of allylthiourea (71).

Another sensitized photooxidation is that of phenylhydrazine by methyl red (73, 128). In this work it is shown that not all forms (ionic species) may take part, but only one, the zwitterion, or, as Livingston suggests, a non-ionic tautomer. This reaction, in which quantum yields up to 0.5 may be obtained, is independent of the chlorophyll concentration and, per unit of light, of the light intensity over a large range.

That chlorophyll photooxidation may be accelerated by ascorbic acid and inhibited by hydroquinone was shown by Aronoff and Mackinney (*loc. cit.*).

MECHANISM OF FORMATION OF CHLOROPHYLL AND RELATED COMPOUNDS

We are now reasonably certain that the immediate precursor of chlorophyll is protochlorophyll. We have almost no idea what the precursors, especially the colorless ones, of protochlorophyll are.

The speculations to be presented may not have real significance biochemically, but they have played a part in the repertoire of plant physiology. It is, therefore, not entirely wasteful to summarize, coordinate and evaluate these theories. There will, of course, come a time when our only direct means of solution, i.e., isotope and/or mutant investigations, will have to envisage these mechanisms in their larger aspect.

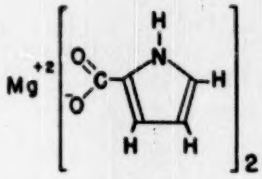
Theories concerning the mechanism of chlorophyll formation may be divided into three classes: *a*) speculations concerning the over-all method of formation up to a molecule as protochlorophyll; *b*) initial isotope experiments dealing with the utilization of metabolic intermediates, as glycine and acetate, in hemin synthesis, and which may not be directly applicable to chlorophyll; and *c*) physiological observations and correlations pertinent to chlorophyll formation.

Despite the wide-spread nature of the porphyrins, including chlorophyll, relatively little is known concerning the mechanism of their formation. It is known, for example, that iron is required, not only because of its intrinsic nature in the respiratory enzymes and hemoglobin, but also for chlorophyll formation in plants. Similarly Cu is necessary for both chlorophyll and hemoglobin formation. Until recent introductory isotopic experiments, nothing at all was known of the possible organic intermediates in its formation, for neither pyrrole[6] nor any of its substituted compounds from which porphyrins may be synthesized are found in nature. The question of the mechanism of porphyrin formation must also solve an additional problem, that of absence of some of the possible isomers (generally, 2 of 4) and predominance of one of those which do exist. More specifically it concerns the four isomeric types, corresponding to etioporphines I to IV, only types I and III of which occur in nature, and of these, type III predominates,

[6] Occasionally, as in the work by Troensgaard (223), one finds reports of pyrrole rings in proteins. Since it is well known that dry distillation of various substances, e. g., proteins, glutamic acid, etc., yield pyrroles (witness the process by which pyrroles are (were) manufactured by dry distillation of bones)—efforts must be made to prove their natural occurrence.

except under some pathologic conditions. Theories which have been advanced regarding porphyrin formation will be considered first.

THE IRON-PYRROLE THEORY. A theory advanced (173) in 1915 enunciated the role of Fe in plants as that of producing pyrrole derivatives from non-cyclic compounds which follow some undetermined path to chlorophyll. Evidence for this consisted in the greening of Fe-chlorotic plants which had been fed Mg-pyrrole-2-carboxylate (IV):



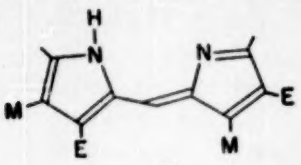
IV

Because of objections to this theory, two additional papers confirming the original work were published in 1935 (172, 174) and still another in 1938 by Lodoletti (129) who substituted 2-acetyl pyrrole for the carboxylic derivative with identical results and claimed an extension of the theory to any pyrrole derivative, such as acetyl-methyl-indole and acetyl-methyl-benzopyrrole, despite their insolubility in aqueous solution.

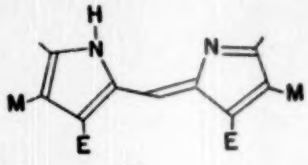
Deuber (43), using some pyrrole-2-COOH obtained from Oddo in addition to his own, not only was unable to obtain greening but found definite toxicity at lower levels than were specified for the original experiments. Similarly Demidenko (42) and Godnev (75) were unable to confirm the theory. The author (9) has found that neither pyrrole-2-aldehyde nor pyrrole-2-COOH can substitute for Fe in chlorophyll formation; nor can sodium bilirubinate, tryptophane, succinimide or mesoporphine serve in that capacity.

On feeding pyrrole-2-aldehyde to either *Chlorella* or barley roots (9), a purple pigment is developed. It is possible that this pigment is a porphyrin and that plants do have the capacity to produce porphyrins from pyrroles. It is, however, also certain that if the function of iron is to produce pyrroles, these latter are not in themselves sufficient to produce chlorophyll.

THE PYRROMETHENE THEORY. That porphyrins may arise from pyrromethenes has been postulated on two accounts, *a*) because of the terminal degradation of hemin in the animal to pyrromethenes, and *b*) because it offers an explanation of the dualism of the porphyrins in nature (i.e., types I and III[7] on the basis of etioporphine). Thus Dobriner (44) proposed two types of pyrromethenes (V and VI) which yield porphines of types I, II or III,



V

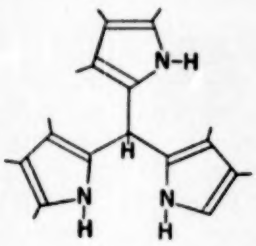


VI

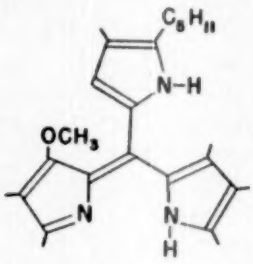
M = methyl
E = ethyl

depending upon whether they combine with themselves or with each other. However, neither etioporphine II nor its derivatives have been found in nature. The manner of origin or of the coupling of the pyrromethenes is not discussed.

Turner (224) suggests a manner in which different pyrromethenes may arise from a single source, namely, a tripyrrylmethane (VII), akin to the pigment of the bacterium *Bacillus*



VII



VIII

prodigiosus (VIII). By rupture of any one of the three carbon-nitrogen bonds, three different pyrromethenes may be formed.

[7] The four etioporphines are:

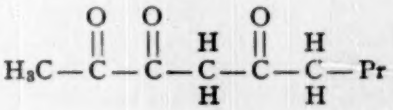
Type I	: 1,3,5,7–	methyl,	2,4,6,8–	ethyl porphine
" II	: 1,4,5,8–	" ,	2,3,6,7–	" "
" III	: 1,3,5,8–	" ,	2,4,6,7–	" "
" IV	: 1,4,6,7–	" ,	2,3,5,8–	" "

Turner further suggests that tryptophane may be the initial compound which, following a series of oxidations, may be transformed to a pyrrole.

A number of difficulties are involved in this theory, e.g., one of the indicated ruptures leads to a pyrromethene, condensation of which leads to type II etioporphines. Presumably one could say that in the organism this manner of rupture is enzymatically prevented. Secondly, although dipyrromethenes are well known in nature as breakdown products of bilins, tripyrromethenes are not. Indeed the bacterial pigment mentioned above is the sole example of its type known in living matter. Thirdly condensation of pyrroles to tripyrrylmethanes must also be viewed with some doubt inasmuch as, as previously mentioned, single pyrroles (not pyrrolidines) have not as yet been found in nature.

THE CARBOHYDRATE THEORY. Thomas (222) also considered the possibility of the oxidation of tryptophane to pyrroles in porphinogenesis. He showed quite definitely, however, that tryptophane was unable to generate protoporphine in the Harderian gland of the rat. [It did exert an apparent excitatory effect on hemoglobin formation, as noted by a number of others (1, 93, 138).] Nor did proline or oxyproline increase the glandular porphine. By utilization of various types of diets he reached the conclusion that porphyrin formation is for the most part effected by simple carbohydrates without specific characteristics, and not by particular substances. That is to say, the metabolism of carbohydrates provides building units for porphine synthesis, and these units are not as yet known.

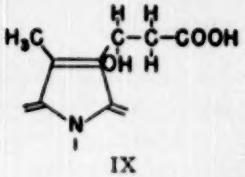
It is a well-known fact that animal tissues are generally unable to synthesize aromatic units, as the benzene ring, thus resulting in, e.g., the indispensability of some of the amino acids. Thus a variety of theories have been proposed in which sugars and sugar derivatives have been made the basis of porphine synthesis. For example Robinson (183) has suggested the combination of a "hexose" and a "triose" (in an unspecified manner) to a high state of oxidation, resulting in the compound:



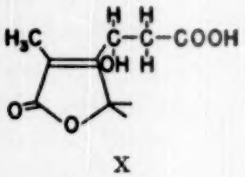
Four of these units are then combined, and, following addition of nitrogen via ammonia, dehydration and two isomerizations, a vinyl porphine may be obtained.

It is of course a simple matter to show on paper (8) that chlorophyll may be divided into four identical units (IX) whose propionic acid residues may be transformed by relatively simple biological reactions to the known residues of chlorophyll.

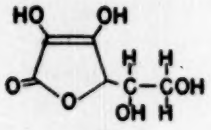
It is interesting to speculate that the initial condensing material is not of the pyrrole type, but of the lactone. This, then, suggests a moiety (X) similar to that of ascorbic acid (XI) from which pyrroles can be made readily via the well-known ammonium pyromucate synthesis of pyrrole itself.



IX



X



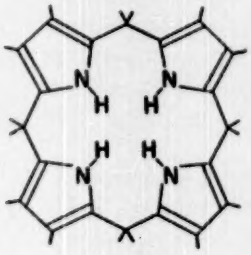
XI

Correlations between ascorbic acid content of leaves and the amount of chlorophyll at various stages and conditions have frequently been noted (59, 81, 143, 181, 225). This does not imply that ascorbic acid itself is directly involved in chlorophyll synthesis, although it may play a dual role. For example, Gortikova and Sapozhnikov (81) have shown the dependence of greening in plants upon the redox potential. By addition of ascorbic acid in optimum amounts at various pH levels to etiolated leaf blades in the dark, followed by exposure to light, a maximum amount of chlorophyll was formed at pH 8.2. Other substances, as 2,6-dichlorophenol indophenol, may substitute for ascorbic acid in regulating the redox level. Using a variety of redox indicators, the rH maximum for chlorophyll formation was found to lie between 17 and 22. One may observe that a lactone of the type of ascorbic acid may serve in any or all of three manners: *a*) in an indirect

manner: dehydroascorbic acid + oxidizing enzyme ≡ ascorbic acid + reduced enzyme, *b*) directly, by conversion of the lactone to a pyrrolone, *c*) coincidentally, i.e., as an index of the degree of oxidation of sugars (e.g., to diketones or to lactones) necessary for the formation of porphyrins.

THE CAROTENOID THEORY. Von Euler (58) has suggested that the conjugated carbons of the carotenoids provide the framework of the porphyrin nucleus. It is a fact that carotenoids always accompany chlorophyll, although the reverse is not true. While it is more probable that there is a relationship between phytol and the carotenoids than between the latter and chlorophyll, there is as yet no evidence that they bear any closer relationship than that of a common origin.

THE PORPHYRINOGEN THEORY. Godnev showed some time ago (76, 78) that it is possible to reduce protochlorophyll extracts with zinc and then reoxidize them in air to a green solution of identical absorption spectrum. He thus concluded that within the plant protochlorophyll arises from a colorless precursor of a character similar to the reduced protochlorophyll, probably a porphyrinogen (XII). It is a simple matter to form porphyrinogens chemically,



XII

although their oxidation to porphines proceeds only with difficulty and in poor yield. Smith (214) has shown, however, that there are ether-soluble, colorless, magnesium compounds in leaves, and it would be interesting to know, as a beginning, whether or not they also contain nitrogen.

Indeed Granick (84, 85) has obtained orange-colored mutants (grown in light or dark) which contain protoporphine and Mg protoporphine, respectively, as their primary porphine pigments.

The interpretation of this is that in the production of the mutants from the wild type by UV, a gene in the sequence required for chlorophyll formation has been destroyed. Presumably this is the gene synthesizing the enzymes which convert in the first instance, protoporphine to Mg protoporphine; and in the second instance, Mg protoporphine to a precursor of protochlorophyll. Indeed, this precursor of protochlorophyll, Mg vinyl pheoporphine a_5 (protochlorophyll minus phytol), has recently also been found in very small quantities in another mutant (85*a*). A summary of the sequence of known compounds involved in chlorophyll formation is given by Granick as:

$$\text{glycine} + \text{acetate} \longrightarrow \text{protoporphine IX (heme minus Fe)} \xrightarrow{\text{Mg}}$$
$$\text{Mg vinyl pheoporphine } a_5 \xrightarrow{\text{4 or 5 steps}} \text{Mg protoporphine IX} \xrightarrow{\text{phytol}}$$
$$\text{protochlorophyll} \xrightarrow{2\text{H} \pm h\nu} \text{chlorophyll.}$$

While this type of reasoning is that normally employed in genetic studies of this kind, it appears also possible that a mutation has occurred which is esentially a rearrangement of a gene, rather than a complete elimination, so that the enzyme formed pursues a somewhat different pathway than that of the wild type enzyme. Under natural conditions for these organisms such a mutation would be lethal, but not under those used to induce growth. Thus while the above compounds may be intermediates in chlorophyll formation, the evidence is not yet conclusive. One must in fact reconstruct the entire chain.

The genetics of chlorophyll formation in corn indicate that there are at least twelve genes involved, and a variety of strains may be obtained with different levels of chlorophyll concentration. As an approach to the problem it is necessary to know whether or not the chlorophylls are identical in these different strains or whether a multiplicity of genes are required for maximum chlorophyll formation. If the latter is the case, then the actual biochemical steps from colorless precursor to protochlorophyll may be less than twelve.

It seems somewhat simpler, however, to approach the problem of chlorophyll formation by the use of organisms which form chlorophyll only in the light (e.g., *Euglena viridis*) or of mutants of wild types which normally form chlorophyll in the dark but now

do so only in the light. Such organisms have recently been described by Davis (41) and by Granick (84, 85). It may well be that with these organisms the fortunate choice of a substrate whose formation was eliminated by mutation may again permit the formation of chlorophyll in the dark and thus provide an insight into the mechanism of chlorophyll formation.

Tuttle[8] has fed labeled (C^{14}) acetate to leaves and found that only the methyl carbon enters into chlorophyll formation. The extent to which the acetate molecule itself enters into the formation of the pigment is not known. As similar experiments (vide infra) with hemin indicate, the acetate may well be metabolized into other substrates used more directly. Recent papers dealing with the mechanism of hemin formation are listed in the bibliography (2, 24, 87, 150, 151, 200, 200*a*).

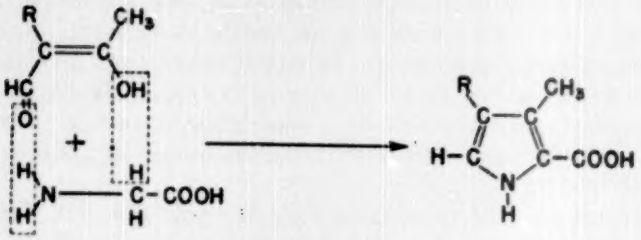
Shemin and Rittenberg utilized glycine containing N^{15}, and fed to rats the following compounds containing labelled N: glycine, ammonium citrate, *dl*-glutamic acid, *dl*-proline, and *dl*-leucine. The feeding was done over a three-day period, following which the normal rations were continued for two weeks before the hemin from the animals was analyzed and the results expressed as the ratio of $\frac{N^{15}\text{ conc. in hemin} \times (100)}{N^{15}\text{ conc. in compound fed}}$. The results for the respective compounds are 0.91, 0.09, 0.17, 0.18, 0.07. It was, therefore, apparent that glycine was the most effective source for the nitrogen of the porphine among the ones used. It did not prove, of course, that other amino acids, e.g., alanine, would not be equal or superior, or, indeed, that other types of nitrogenous compounds also were not effective. Furthermore the glycine may have served merely as an intermediate for rapid metabolism, e.g., transamination.

It has recently been shown (200*b*) that alanine will not substitute for glycine, nor will serine, alanine, aspartic acid or acetyl glycine. Although conversion of serine to glycine is known, the rate of incorporation of the glycine to porphyrin is so much more rapid than the conversion of serine to glycine that it is not possible to "dilute" the radio glycine by the addition of serine.

Blotch and Rittenberg, using deutrioacetate, showed that deuteriohemin was formed, with a D-content of hemin above that of the equilibrium D level of the medium. This indicated that the

[8] Personal communication.

methyl carbon of the acetate, though not necessarily acetate itself, is involved in reactions which cause it to be synthesized into a methine carbon in the porphine ring, or a substituent on one or more of the pyrrole rings, since these positions contain the only hydrogenated carbons in hemin. Because of the known conversion of acetate to glycine, a reaction mechanism for synthesis of the pyrrole ring was given as



This mechanism is presumably intended to apply only to the chemical nature of the intermediates; otherwise the mechanism itself is open to the objection given above for individual pyrroles.

Grinstein *et al.*, in extending the above experiments, used carboxyl-labeled glycine and could not find evidence that it was used in hemin synthesis. Altman *et al.*, however, using methyl-labeled glycine, did find it to be preferentially used in hemin synthesis. Because there is no unequivocal evidence that the carboxyl-carbon of glycine (or acetate) is involved in hemin synthesis, it becomes necessary to *a*) label all three atoms involved, i.e., to synthesize, e.g., deuterio, carboxy C^{14}, N^{15}-glycine and determine whether all are incorporated in hemin in the same ratio, and *b*) to degrade hemin, e.g., as described in the porphine degradations of chlorophyll chemistry, and find where in the molecule the glycine or its parts are involved.

More recent work (87*a*) with C^{14}-labeled glycine has proved that the carboxyl carbon is not incorporated into the hemin, though it is into the protein, nor is there exchange of such carbon from the protein to the hemin. Thus it is evident, as these authors point out, that only the $-\overset{H}{\underset{H}{C}}-NH_2$ part of glycine is utilized for hemin formation. There still remains to be proved that this group enters intact.

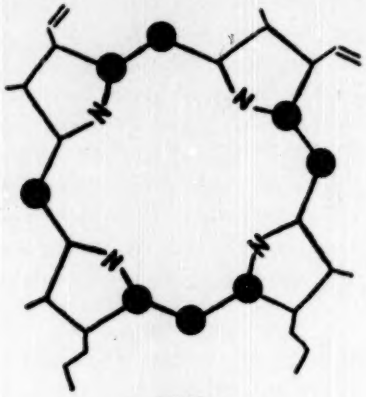
Such evidence has recently been provided for hemin by Muir and Neuberger. These workers used not only labeled glycine, but also ethanolamine, which they believed might be one of the metabolic products derivable from glycine. The ethanolamine was incorporated only poorly into porphine. They established the conditions for the chromic acid oxidation of mesoporphine[9] on a semi-micro scale. In this isolation the N's of rings I and II are indistinguishable, as are those of III and IV. With the methylene-labeled glycine, the N's in the two groups of rings were found to be identical. On the basis of the atom % of the extracted hemin, taking into account its rate of formation, and the N^{15} content of the glycine pool, it was believed that the N^{15} must be incorporated into all the N's and therefore not only that $N_I + N_{II} = N_{III} + N_{IV}$ but that probably $N_I = N_{II} = N_{III} = N_{IV}$.

In an important abstract (151) Muir and Neuberger report results of the continued study of hemin formation, using doubly-labeled glycine ($N^{15}H_2-C^{14}H_2-COOH$) and labeled acetate ($CH_3-C^{14}OOH$ and $C^{14}H_3-COOH$). The oxidation products of mesoporphine, itself derived from hemin, are hematinic acid (rings III and IV), methylethylmaleic imide (rings I and II) and $BaCO_3$ (primarily methine carbons). With the labeled glycine, and assuming all the porphyrin nitrogen to arise from the glycine, it was found that $\dfrac{\dfrac{(C^{14})}{(N^{15})}\text{ porphyrin}}{\dfrac{(C^{14})}{(N^{15})}\text{ glycine}} = 2.4$. Further the specific activity of the imides was equal and about twice that of the $BaCO_3$. It is thus apparent that the methylene carbon of glycine can be incorporated into both ring- and methine-carbons of hemin. It is also apparent that glycine is further metabolized in some manner so as to lose some of its nitrogen prior to incorporation into the hemin. With the methyl-labeled acetate, incorporation of carbon into the rings is approximately equal for all rings, but does not enter at all into the methine carbons. Finally, with carboxyl-labeled acetate, although practically no incorporation into methine carbon occurs, utilization into the rings is unequal, the specific activity of rings III and IV being about four times as great as

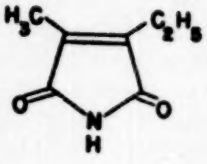
[9] [Mesoporphine IX = 1,3,5,8-tetramethyl, 2,4-diethyl, 6,7-propionic acid porphine. The mesoporphine is obtained by catalytic reduction of protoporphine; the latter is hemin minus Fe.]

those of rings I and II. A comparison of the incorporation of carboxyl versus methyl-labeled actate (both hemins were initially of similar specific activities) for the two groups of rings shows roughly the indicated values of Table II. Since rings III and IV of hemin contain propionic acid residues, it is reasonable to conclude that the carboxyls of the acetate are transformed into the terminal carboxyls of the propionic acid.

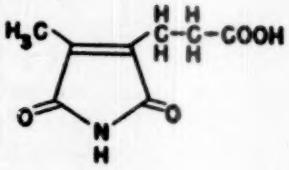
In a more recent abstract Wittenberg and Shemin (234*a*) state that α-carbon of glycine enters into the porphyrin molecule at the positions indicated in the diagram. The carbons of acetate are said to account for "most of the rest" of the positions. Details are not yet available.



XIII
heme skeleton



XIV
methyl ethyl maleic imide



XV
hematinic acid

Chlorophyll also contains a propionic residue on C_7 and an equivalent structure on C_8, i.e., the isocyclic ring. The failure of Tuttle

to obtain incorporation of carboxyl-labeled acetate is therefore not clear. Again, reasoning from Table II, assuming the validity of a direct quantitative comparison, one would infer that the methyl carbons became the α and α' carbons of all the rings. It is pure speculation to assign positions to the carboxyl carbons, aside from the carboxyl of the propionic acid residue in rings III and IV. In rings I and II, however, one presumes they are associated with the vinyl groups.

Connected with formation of chlorophyll is the problem as to whether or not there is a daily variation, which has been reported by a number of workers, often with disregard for the wet weight (e.g., 30, 31, 189). Others (e.g., 37) imply a similar state when the notion of constant (usually implied as rapid) formation and destruction of chlorophyll exists. That rapid change does not exist can be shown by a calculation of the specific activity of the chloro-

TABLE II

	relative activity of	
	III & IV	I & II
carboxyl-labeled acetate	8	2
methyl-labeled acetate	4	4

phyll formed during photosynthesis of barley with radioactive carbon dioxide (11) which is, e.g., about 1/1000 that of the sugar formed. Later experiments (33) have shown that incorporation of both C^{14} and D into chlorophyll of *Chlorella* proceed in the ratio required by the empirical formula but less than that demanded by the composition of *Chlorella*. Net formation of chlorophyll thus appears to proceed faster in the dark than in light in these organisms.

Zaitseva (239) has shown that the removal of the endosperm from wheat seedlings diminished the amount of chlorophyll formed in light only if external sucrose was not supplied. A source of chemical energy is thus required. As mentioned earlier, Gortikova and Sapozhnikov (*loc. cit.*) have shown a dependence of chlorophyll formation, in both rate and amount, upon the redox potential which is determined by regulation of the pH and by ascorbic acid.

The claim is indeed made that the ascorbic acid state thus regulates chlorophyll formation. Oxygen, which is also known to be prerequisite for chlorophyll formation, could, however, be replaced by an organic hydrogen acceptor, as 2,6-dichlorophenol-indophenol.

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